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14. ABSTRACT

Invariant natural killer (iNKT) cells are a unique population of immune cells that rapidly secrete a variety of cytokines upon activation and have been generally attributed potent anti-tumor functions. However, our studies using the murine 4T1 breast cancer model indicate a regulatory/suppressive function that markedly inhibits effector response generated by combined radiotherapy and immunotherapy to either CTLA-4 or 4-1BB (CD137), resulting in dramatic improvement in survival in NKT-/- mice. Our data also indicate that regulatory iNKT cells may act at the level of antigen presentation since NKT-/- mice were found to have significantly more dendritic cells of a highly mature phenotype than wild-type tumor mice. Regulatory function could not be rescued by in vivo administration of a strong Th1-inducing NKT agonist α-galactosyleramide; we hypothesize that blocking antibodies to CD1d (clone 20H2) will relieve this immunosuppression. We have spent considerable time in ensuring that the blocking antibody will not have any secondary effects that include depletion or stimulation of CD1d-expressing DCs. Studies are now underway to test whether blocking this interaction will abrogate the suppressive effect of iNKT cells on therapy-induced anti-tumor immune response.

15. SUBJECT TERMS
invariant natural killer T cells, breast cancer, immunology, dendritic cells, 4T1 tumor

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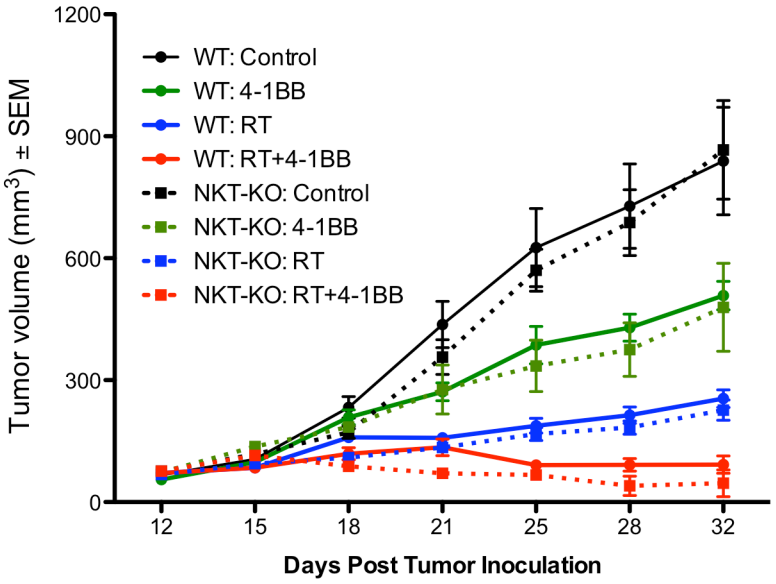
INTRODUCTION

Invariant natural killer T (iNKT) cells comprise a unique group of immune cells that specifically recognize lipid antigens presented in the context of CD1d molecules which leads to rapid and robust secretion of a wide range of Th1 and Th2 cytokines (Kronenberg, 2005). Although iNKT cells represent a small population of cells, their role in shaping the ensuing adaptive response puts them at a critical bridge between the innate and adaptive immunity. In cancer, iNKT cells are generally attributed a role in tumor immunosurveillance (Nishimura et al., 2000; Tomura et al., 1999) . However, using the 4T1 mouse model of metastatic breast carcinoma, we have seen that they assume a largely regulatory function especially in downregulating the therapeutic response to our regimen of combined local radiation and immunotherapy with CTLA-4 blockade (Pilones et al., 2009). It is likely that this regulatory function is imparted both by the host immune cells as well as tumor-derived factors. The goal of this project is to test the hypothesis that iNKT cells can be conditioned by the tumor microenvironment to switch to an immunoregulatory phenotype which inhibits the generation (priming) or effector function of anti-tumor T-cells. Three non-mutually exclusive hypotheses will be tested using the 4T1 model: 1) 4T1 tumor cell express a yet unidentified lipid antigen that, when presented directly or indirectly by dendritic cells to iNKT cells, can induce preferential secretion of immunosuppressive cytokines ; 2) iNKT cells inhibit effector T cell priming by killing dendritic cells that cross-present 4T1-derived tumor antigen and 3) immunoregulatory iNKT cell, by acting directly or indirectly through the secretion of cytokines, promote the generation and maintenance of regulatory T-cells.

BODY

We have previously shown that NKT cells can have an inhibitory effect on anti-tumor immunity generated by combined radiotherapy and anti-CTLA4 immunotherapy (Pilones et al., 2009). This conclusion was given support by several key findings, including: 1) 4T1 tumors grew with comparable efficiency in both WT and NKT-deficient mice (NKT-/-) but NKT-/- mice had significantly fewer lung metastases, indicating an improved spontaneous effector response ; 2) the spontaneous effector response seen in NKT-/- mice was CD8-mediated and not the result of intrinsic immunogenicity and 3) an impressive 50% of NKT-/- mice were able to completely reject tumor growth and mount a successful anti-tumor memory response to re-challenge when given the combined therapy of local radiation and checkpoint blockade (anti-CTLA4) immunotherapy. In the past year, an additional experiment was performed to determine whether the differential response was immune therapy-specific. Agonistic anti-4-1BB (CD137) antibodies (BMS-469492, Bristol-Myers-Squibb) were used in conjunction with radiotherapy in well-established 4T1 tumors in WT and NKT-/- mice. 4-1BB is a co-stimulatory molecule that is stably upregulated on activated T-cells and provides survival signal that promotes expansion, upregulation of anti-apoptotic genes and cytokine production. Survival data shown in figure 1 show as much as 50% NKT-/- mice were able to completely reject 4T1 tumors compared with 20% long-term survival in WT mice. The data indicate that, in the 4T1 model, the inhibitory effect of NKT cells on therapy-induced anti-tumor response is not specifically dependent on the method of immune stimulation.

A.



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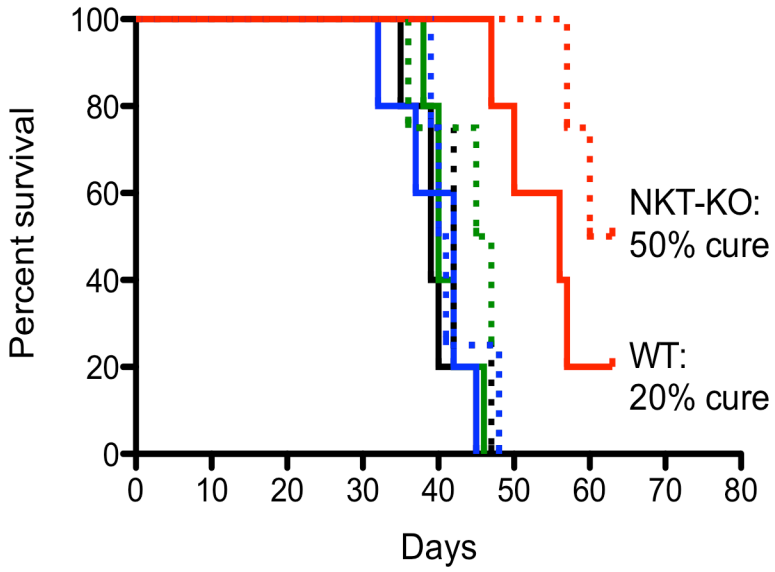


Figure 1: Groups of 4-5 WT and NKT-/- mice were inoculated s.c. with 4T1 tumors on day 0. Some mice were given 2 doses of 12Gy local radiation on days 13 and 14 post tumor inoculation. Agonistic α-4-1BB antibodies (200 ug/mouse) were given i.p. 1,3 and 5 days after the last radiation dose. Mice were subsequently followed for tumor growth (A) and survival (B).

Specific Objective 1: 4T1 tumor cell express a yet unidentified lipid antigen that, when presented directly or indirectly by dendritic cells to iNKT cells, can induce preferential secretion of immunosuppressive cytokines

In the last progress submission, we reported that 4T1 breast cancer cells , despite low-level expression of CD1d, can present aGalCer on their surface, and stimulate production of IL-2, IL-4 and IFN-γ from DN23.D3 NKT hybridoma cells. We have done additional experiments to show that this response is CD1d-mediated, since a blocking antibody (3C11 mAb) can abrogate the cytokine response in a dose-dependent manner (Figure 2).

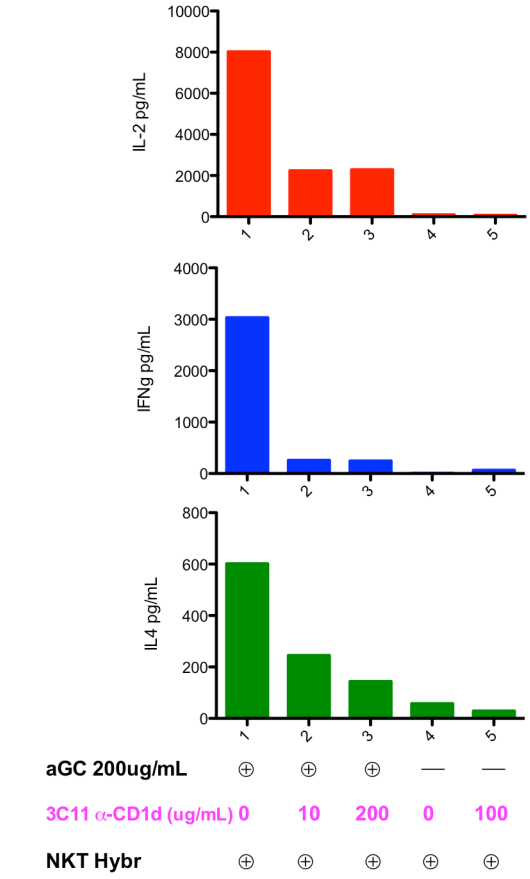


Figure 2: Tumor cells can activate NKT cells in a CD1d-dependent manner. Irradiated 4T1 cells were loaded with aGalCer or vehicle overnight and wash prior to incubation with anti-CD1d (3C11 mAb). After 24 hours, cells were washed extensively, fixed in 2% p-formaldehyde and cultured with DN32.D3 NKT hybridoma cells. Secreted IL-2, IL-4 and IFNγ in the supernatant were measured by Flowcytomix beads (eBioscience).

Specific Objective 2: iNKT cells inhibit effector T cell priming by killing dendritic cells that cross-present 4T1-derived tumor antigen

In the second year, we proposed to execute experiments that specifically address whether the immunoregulatory NKT cells work at the level of cross-priming. We have found quantitative differences in dendritic cells infiltrating 4T1 tumors and in tumor-draining lymph nodes (Figure 3A, 3B) between WT and NKT-/- mice that indicate NKT cells directly target dendritic cells (DC) crucial for cross-priming of effector T cells. The quantitative differences could not be attributed to strain-specific factors since the DCs in lymph nodes of healthy mice were comparable in both strains (Figure 3C).

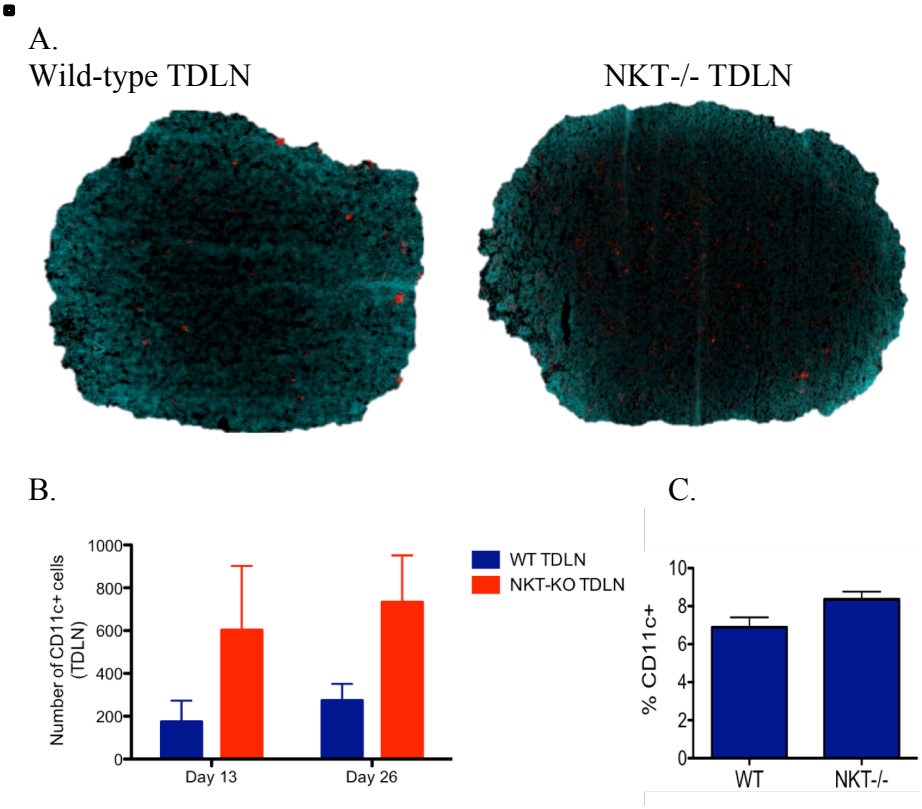


Figure 3: Quantitative differences in DC numbers in tumor-draining lymph nodes (TDLN). 4 mice from each strain were injected s.c. with 4T1 tumors and TDLN harvested on days 13 and 26 and frozen in OCT. Sections were stained for CD11c and number of cells were counted using ImageJ (NIH). (A) Representative stained tissue sections from TDLN of tumor-bearing WT and NKT-/- mice. (B) Summary of DC counts from TDLN. (C) Summary of DC counts from healthy mice.

NKT cells are generally considered to be strong inducers of anti-tumor immunity, especially when stimulated by pharmacologically strong agonists such as α-galactosylceramide (aGalCer) (Fujii et al., 2002; Shimizu et al., 2007). However, NKT cells can likewise assume a regulatory role instead, as supported by our published studies in the 4T1 model. In such a scenario, stimulation by aGalCer has not been found to be afford any therapeutic benefit and may even theoretically enhance their suppressive effects. Thus, new strategies are needed to abrogate pathways through which NKT cells exert negative regulatory functions. To this end, we investigated whether blocking the interaction of NKT cells and DCs by blocking CD1d can abrogate NKT immunoregulation and further enhance anti-tumor immune response generated by combined radioimmunotherapy. For these studies, we used the 20H2 clone (developed by A. Bendelac , Univ of Chicago) that was shown to have potent blocking activity based on antibody cross-blocking experiments. To our knowledge, the 20H2 clone has not been used for in vivo blocking studies and the available data showing its blocking activity is sparse. Therefore, we have conducted several confirmatory studies that specifically address the following: 1) that the 20H2 clone can block CD1d in vitro ; 2) that in vivo administration of 20H2 does not lead to depletion of CD1d-expressing cells and 3) that the 20H2 will not induce reverse signaling, i.e, lead to stimulation of CD1d-expressing cells especially DCs. These experiments are absolutely critical to ensure correct interpretation of results if 20H2 is to be used solely to block the activation of regulatory NKT cells. The results of these studies are outlined below:

A) 20H2 CAN BLOCK CD1D IN VITRO

We confirmed the blocking activity of 20H2 in vitro, using bone marrow-derived DCs (BMDCs) pre-loaded with aGalCer and cultured with DN32.D3 NKT hybridoma cells (obtained from A. Bendelac). DN32.D3 cells have been shown to recapitulate the cytokine responses of native NKT cells following activation by aGalCer (REF) and have been used extensively by many investigators as a reliable readout for NKT responses (REF). Indeed, we found a dose-dependent inhibition in the production of IL-2, IL-4 and IFN-γ in the supernatants, indicating that 20H2 can block presentation by BMDCs (Figure 4A). We also extended these studies to 4T1 cells (Figure 4B) , which we have shown to express low levels of CD1d in both mRNA and on the surface.

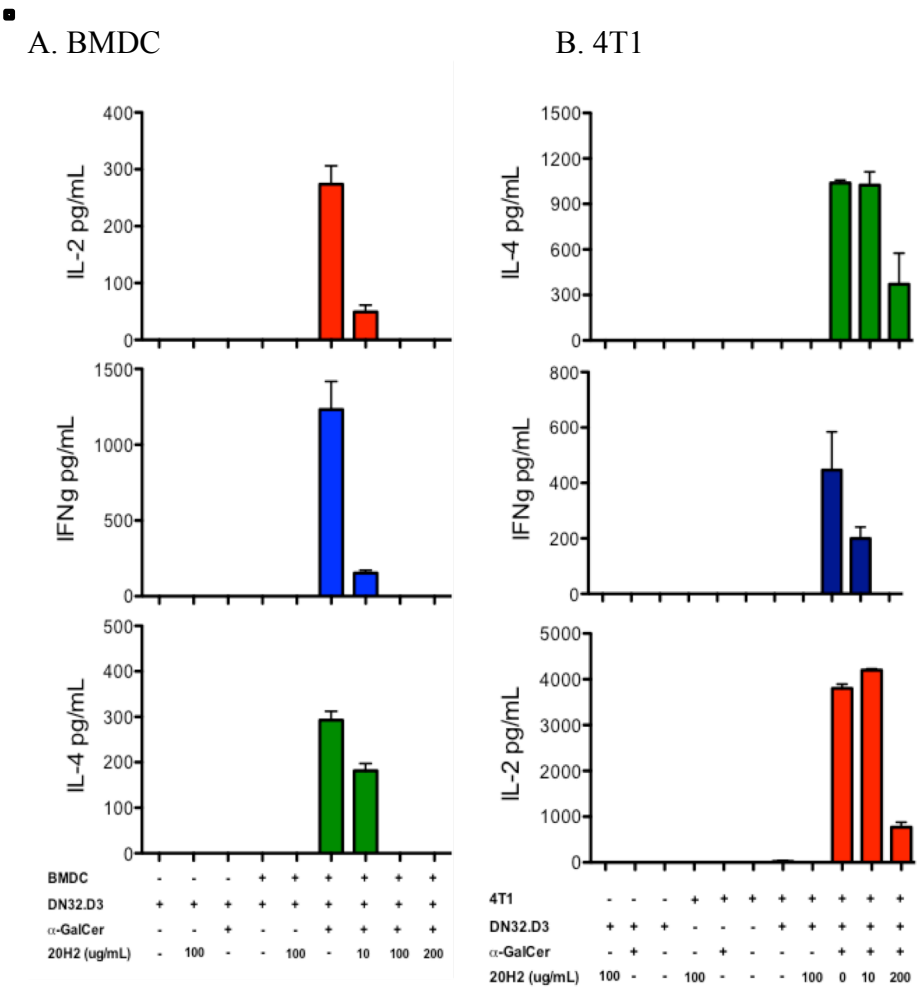


Figure 4. In vitro blocking activity of 20H2. (A) LPS-matured BMDCs or (B) 4T1 were cultured in the presence or absence of 20H2 prior to loading with aGalCer. Feeder cells were extensively washed prior to co-culture with DN32.D3 NKT hybridoma cells.

We further confirmed the effect of 20H2 in inhibiting the proliferation of DN32.D3 NKT hybridoma cells in an in vitro proliferation assay (Figure 5).

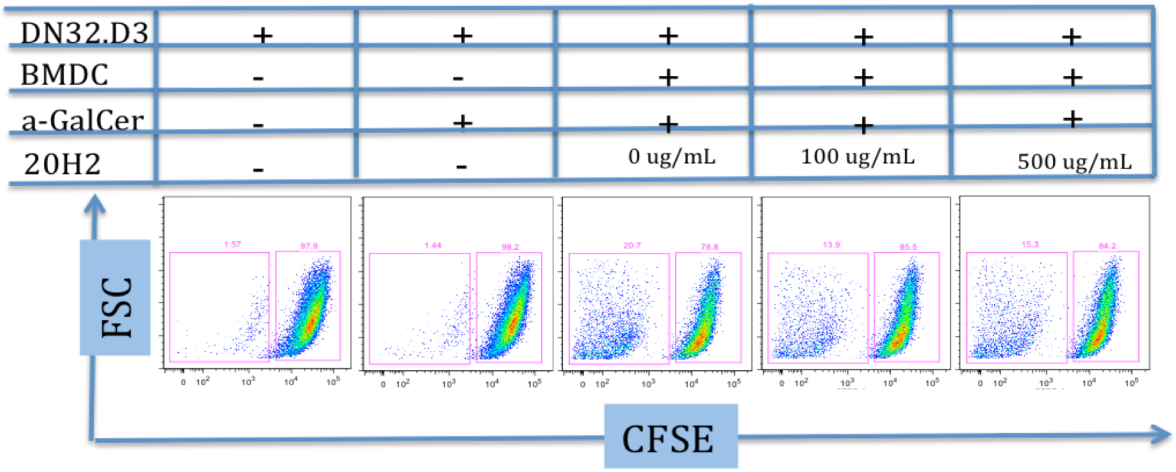


Figure 5: 20H2 can inhibit proliferation of DN32.D3 NKT hybridoma cells. LPS-matured BMDCs were cultured in increasing concentrations of 20H2 prior to loading with aGalCer. Feeder cells were then used to stimulate CFSE-labeled DN32.D3 NKT hybridoma cells. Proliferation was measured after 3 days.

B) 20H2 DOES NOT DEPLETE CD1D-EXPRESSING CELLS

We wanted to confirm whether an intensive dosing regimen of 20H2 will modify expression of CD1d or lead to depletion of cells expressing CD1d in vivo (principally DCs, macrophages and B-cells). Groups of mice were i.p. injected with 200 ug 20H2 or PBS buffer every 4 days for a total of 4 doses. Flow cytometric analysis was done in the spleen to determine quantitative differences in the numbers and percentages of dendritic cells, macrophages or B-cells. The results indicate that splenocyte counts were similar for mice given 20H2 or PBS buffer (Figure 6). Furthermore, intensive dosing with 20H2 did not deplete CD1d-expressing DCs (CD11c+), macrophages (F4/80+) or B-cells (CD19+) (Figure 7).

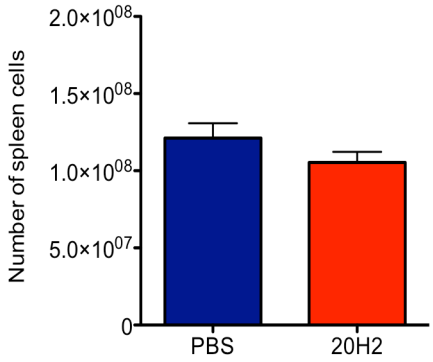
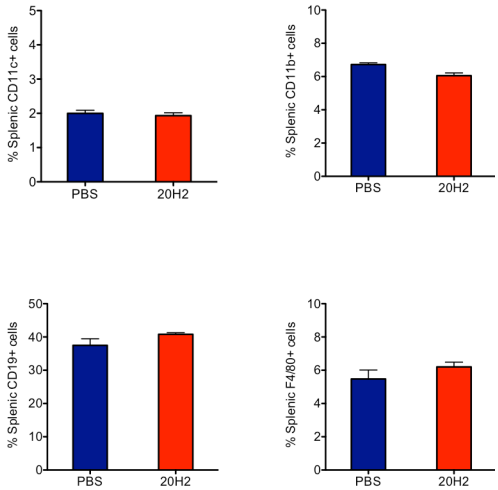


Figure 6. In vivo 20H2 injection did not affect total number of cells in the spleen. Groups of mice (n=5) were i.p. injected with 4 doses of 200ug 20H2 or PBS buffer every 4 days. Spleens were harvested and digested with collagenase and enumerated.

A. Percentages



B. Cell numbers

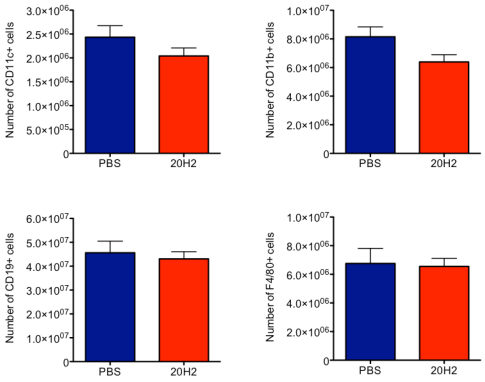
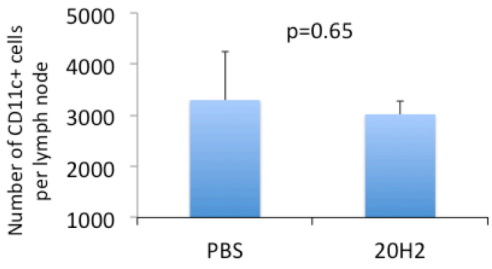


Figure 7: 20H2 Does not deplete CD1d-expressing cells. Groups of mice (n=5) were i.p. injected with 4 doses of 200ug 20H2 or PBS buffer every 4 days. Spleens were harvested and digested with collagenase prior to staining with CD11c-PE-Cy5, CD11b-PE, F4/80-PE or CD19-PE-Cy5. Percentages (A) and cell numbers (B) of DCs were identified by CD11c+CD11b- cells, macrophages by F4/80 and B-cells by CD19.

Since DC comprise the majority of CD1d-expressing cells and play a crucial role in cross-priming of anti-tumor effectors, we confirmed by immunohistochemistry that 20H2 does not deplete the number of CD11c+ cells in the lymph nodes (Figure 8)

Figure 8: 20H2 does not deplete DC in the lymph nodes. Groups of mice (n=5) were i.p. injected with 4 doses of 200ug 20H2 or PBS buffer every 4 days. Lymph nodes were individually harvested and fixed in OCT media. Frozen tissue sections were stained for CD11c+ DCs. Counts were obtained from each lymph node.



We also confirmed by flow cytometry that CD1d expression among DC, macrophages and B-cells remained unaltered with 20H2 exposure (Figure 9).

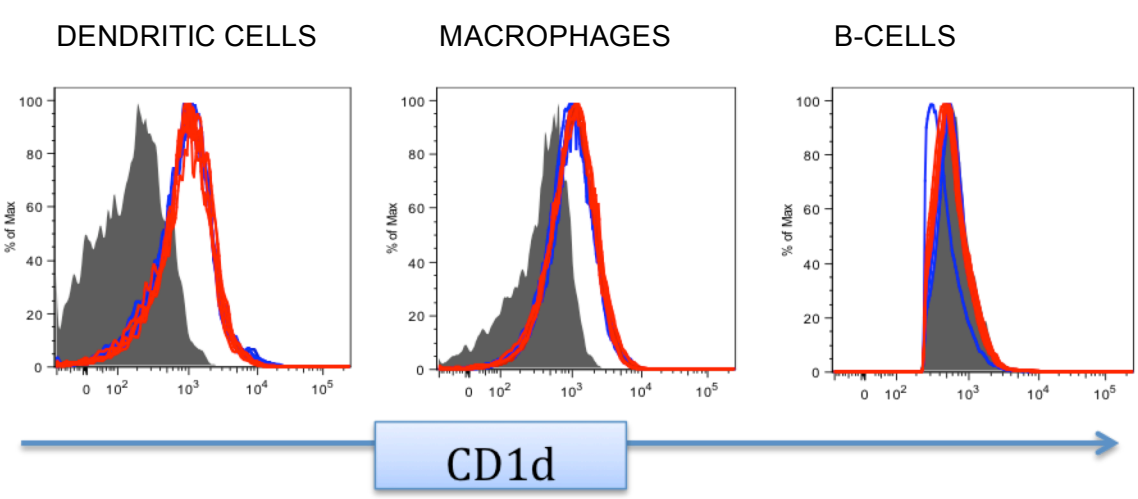


Figure 9. 20H2 does not deplete CD1d-expression in splenocytes. Groups of mice (n=5) were i.p. injected with 4 doses of 200ug 20H2 or PBS buffer every 4 days. Spleens were harvested and digested with collagenase prior to staining with CD1d-FITC, CD11c-PE-Cy5, F4/80-PE or CD19-PE-Cy5. Isotype staining (shaded histogram), and CD1d staining in PBS (blue) or 20H2-treated (red) are shown.

C) 20H2 DOES NOT REVERSE SIGNALLING

The phenomenon of reverse signaling, i.e. induction of a stimulatory effect on CD1d-expressing cells instead of blocking, can severely compromise correct interpretation of results if CD1d blocking is to be exclusively used to inhibit regulatory NKT cells. Stimulation of APC via CD1d has been shown to result in secretion of pro-inflammatory cytokines (e.g. IL-12) known to enhance T-cell priming (REF), and anti-CD1d stimulation have been used in several pre-clinical models to show efficacy in tumor rejection when used in combination with other immunotherapeutics (Teng et al., 2009). To investigate whether 20H2 can induce reverse signaling, we performed in vitro studies using splenocytes from WT BALB/c and NKT-/- mice (Figure 10) which clearly shows that 20H2 does not induce the release of any quantifiable cytokine known to modulate anti-tumor response. We further confirmed these results in vivo by injecting 20H2 i.p. in both strains of mice (Figure 11).

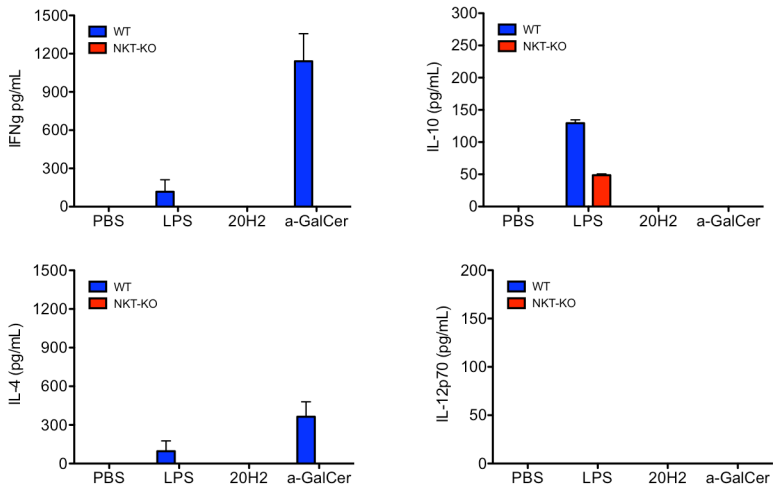


Figure 10: 20H2 does not induce reverse signaling in vitro. Splenocytes ($2x10^6$/well) from WT and NKT-/- mice were stimulated with LPS (100ng/mL), aGalCer (100 ng/mL) or 20H2 (100 ug/mL). Supernatants were collected after 24 hrs for measurement of cytokines by ELISA.

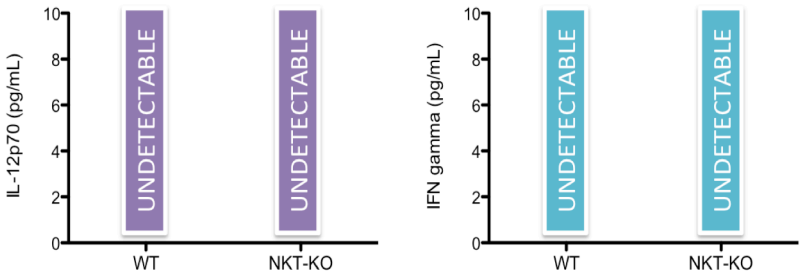


Figure 11: 20H2 does not induce reverse signaling in vivo. WT and NKT-/- mice were injected i.p. with 100 ug 20H2. Sera were collected 24 hours later and assayed fro IL-12p70 and IFNgamma response.

Overall, the results of these studies clearly demonstrate the blocking activity of 20H2 without inducing bystander stimulation or depletion of CD1d-expressing cells. Currently, experiments are being done to evaluate whether 20H2 can abrogate the regulatory effects of NKT cells during the course of 4T1 tumorigenesis and lead to a much improved effector response generated by combined radioimmunotherapy.

Specific Objective 3: immunoregulatory iNKT cell, by acting directly or indirectly through the secretion of cytokines, promote the generation and maintenance of regulatory T-cells.

We have initiated experiments to explore the cross-talk between regulatory phenotypes in the tumor microenvironment, particularly between NKT cells and regulatory T-cells (Tregs). Thus far, we did not see significant differences in systemic and intratumoral populations of Tregs between WT mice and NKT-/- mice (Figure 12).

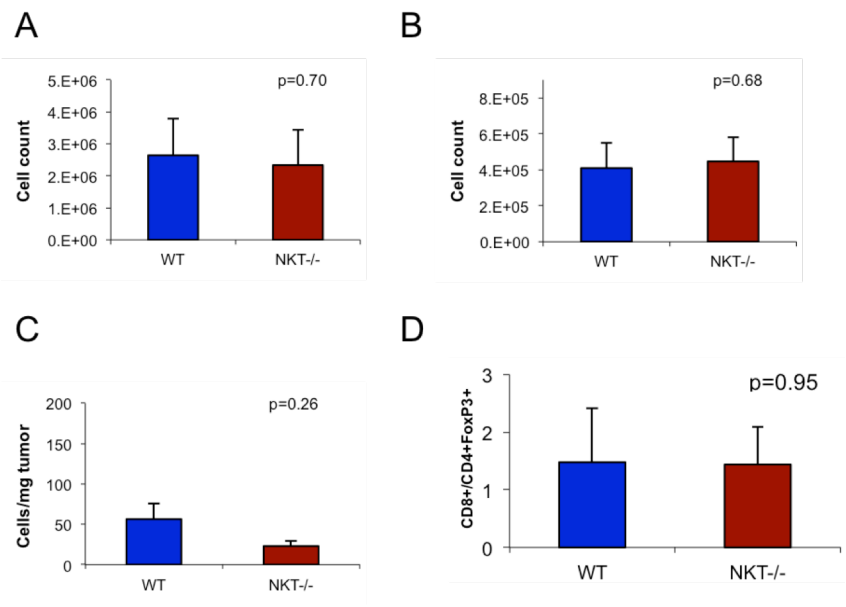


Figure 12: Comparable Treg populations in WT and NKT-/- mice. Groups of mice (n=5) were sc inoculated with 4T1 tumors and sacrificed 21 days later. Cell suspensions from (A) spleen, (B) TDLN and (C,D) tumor were stained for CD4+-FITC, CD25-PE, FoxP3(or isotype)-PE-Cy5. Treg populations were identified by CD4+ cells that stained for both CD25+and FoxP3+. Additionally, effector:Treg ratios in the tumor were calculated (D).

In accordance with the statement of work described for the third and final year, experiments will be conducted to determine whether NKT cells promote the conversion and maintenance of Tregs locally within the tumor microenvironment. For these purposes, we are currently characterizing and optimizing protocols for adoptive transfer of CD4+ T-cells that will allow us to track their conversion into Tregs in the context of a tumor-bearing host.

KEY RESEARCH ACCOMPLISHMENTS

- NKT cells negatively regulate therapeutic response to IR+4-1BB immunotherapy
- 4T1 tumor-bearing NKT-/- mice present with significantly more dendritic cells in the tumor and in the tumor-draining lymph nodes when compared to wild-type BALB/c mice
- anti-CD1d mAb 20H2 can block presentation of CD1d/aGalCer on BMDCs or 4T1 cells and inhibit the activation of DN32.D3 NKT hybridoma cells
- anti-CD1d mAb 20H2 does not lead to depletion of CD1d-expressing cells such as DCs, macrophages or B-cells
- anti-CD1d mAb 20H2 does not induce reverse signaling (i.e., stimulation) in vitro and in vivo
- Treg populations are comparable in both WT and NKT-/- mice bearing 4T1 tumors

REPORTABLE OUTCOMES

A. National Meetings and Poster Presentations

1) Era of Hope Conference
   Aug 2-5, 2011
   Orlando, FL

   Poster P4-13: Immunoregulation of invariant natural killer T cells in a mouse model of metastatic breast cancer
   Poster Session P4: Tumor Immunology

2) NCI Cancer Immunology and Immunotherapy: Building on Success
   Sept 22-23 2011
   Bethesda, MD

3) CTRC-AACR San Antonio Breast Cancer Symposium
   Dec 6-10, 2011
   San Antonio, TX

   Poster P1-01-05: Conditioning by the Tumor Environment Turns Invariant Natural Killer T Cells into Negative Regulators of Anti-Tumor Immunity Elicited by Treatment.
   Poster Session 1: Tumor Cell Biology: Immunology and Preclinical Immunotherapy

B. Institutional Meetings and Conferences

1) NYU Immunology Journal Club
   Meets every Tuesday

2) NYU Cancer Institute Breast Biology Working Group
   Meets every 3rd Wednesday of every month

3) NYU Cancer Institute Immunology Group
   Meets every month

4) NYU Dept of Pathology Works-in-Progress and Journal Club
   Meets every Tuesday
   Last progress presentation: January 2012

C. Mentoring

Joseph Aryankalayil, currently an undergraduate student at NYU

D. Collaborations

1) Mary Helen Barcellos-Hoff, PhD
Associate Professor, Department of Radiation Oncology, NYU School of Medicine

We continue our collaboration with the lab of Dr Barcellos-Hoff on mechanisms of TGF-beta inhibition in cancer, which resulted in a recent publication (see attached appendix). This collaboration has contributed significantly to our understanding of the immunosuppressive mechanisms within the tumor microenvironment. Given the pleiotropic role that TGFbeta plays in downregulating tumor immunosurveillance, it will be of great interest in this project to look at a possible link between TGFbeta and NKT-mediated immunoregulation.

2) Michael Dustin, PhD
Muriel G. and George W. Singer Professor of Molecular Immunology
Co-director, NYU Cancer Institute Cancer Immunology Program

We collaborated with the Dustin lab on a project that studied the cellular basis of anti-CTLA4 (9H10 mAb) immunotherapy. A manuscript was recently submitted detailing these findings.

E. Publications

1). Book chapter:

Sandra Demaria, **Karsten A. Pilones** and Sylvia Adams (2011). Cross-Talk of Breast Cancer Cells with the Immune System, Breast Cancer - Carcinogenesis, Cell Growth and Signalling Pathways, Prof. Mehmet Gunduz (Ed.),
ISBN: 978-953-307-714-7, InTech,
Available from: http://www.intechopen.com/books/breast-cancer-carcinogenesis-cell-growth-and-signalling-pathways/cross-talk-of-breast-cancer-cells-with-the-immune-system

2) Original Research:

Fanny Bouquet, Anupama Pal, **Karsten A. Pilones**, Sandra Demaria, Byron Hann, Rosemary J. Akhurst,Jim S. Babb, Scott M. Lonning, J. Keith DeWyngaert, Silvia C. Formenti, and Mary Helen Barcellos-Hoff. TGFb1 Inhibition Increases the Radiosensitivity of Breast Cancer Cells In Vitro and Promotes Tumor Control by Radiation In Vivo. *Clin Cancer Res*; 17(21); 6754–65.

CONCLUSIONS

Collectively, the data support a key role for dendritic cells in conditioning regulatory NKT cells, and strategies to disrupt suppression by iNKT cells could prove to be therapeutically beneficial in breast cancer. In this aspect, experiments are underway to determine if blocking CD1d can abrogate the regulatory effect of iNKT cells and further enhance effector response generated by combined radiotherapy and immunotherapy. Concurrently, plans are being made to conduct experiments that evaluate aspects of dendritic cell function (i.e, presentation, cross-priming) in greater detail. These experiments, as outlined in the approved statement of work, will comprise much of the work to be done in the third year. We will also be starting work on possible cross-talk between iNKT cells and Tregs.

In the last year, I have actively participated in departmental Works-in-Progress seminars and focused journal clubs (one in immunology and another in breast cancer research specifically) which have enriched my knowledge in cutting edge research in breast cancer. I have had the great opportunity to meet with leaders in the field of breast cancer immunology at recent meetings, and will continue to foster a collaborative relationship with them in the years to come. I continue to work closely with my mentor, Dr Sandra Demaria, who I meet with every week to discuss results and plan experiments. She continues to be an invaluable resource to my training as a future breast cancer scientist.

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APPENDICES

Appendix 1: Sandra Demaria, **Karsten A. Pilones** and Sylvia Adams (2011). Cross-Talk of Breast Cancer Cells with the Immune System, Breast Cancer - Carcinogenesis, Cell Growth and Signalling Pathways, Prof. Mehmet Gunduz (Ed.),

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Appendix 3: Formenti, S, Encouse, G, Adams, S, **Pilones, KA**, Ruocco, MG, Dustin, M and Demaria,S, Role of T-Lymphocytes for Tumour Response to Radiotherapy *Eur J Cancer*; 47(1); S11.

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Cross-Talk of Breast Cancer Cells with the Immune System

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1. Introduction

Understanding the pathogenesis of breast and other cancers requires an improved understanding of the local microenvironment in which cancer develops and progresses (Hanahan and Weinberg, 2011). Many cell types have been defined as key components of the tumor stroma that contributes to tumor growth and metastasis, and modulates the response to treatment. In this chapter we will focus on cells of the immune system, critical players with dual function comprising cells that can foster a pro-tumorigenic inflammatory environment as well as reject tumors (Demaria et al., 2010). Importantly, the therapeutic manipulation of the host immune system has a tremendous potential to enhance the response of breast cancer patients to treatment. Therefore, it is imperative to understand the cross-talk between breast cancer cells and cells of the innate and adaptive immune system.
Several cell communication systems are involved in this cross-talk, including pro-inflammatory and immunosuppressive cytokines, chemokines and endogenous danger signals, known as damage-associated molecular pattern (DAMP) molecules that bind to Toll-like Receptors (TLR). Some of these factors represent interesting targets for immunotherapy strategies based on their known ability to stimulate the immune system, but in the context of the tumor microenvironment these immune stimulatory agents may also produce unwanted pro-tumorigenic effects by binding to receptors ectopically expressed on the cancer cells. Others are involved in recruiting to the tumor immune cells with regulatory and immune suppressive functions that protect the tumor from immune rejection. Clearly, the cross-talk between epithelial cells and the immune system is distorted in cancer to promote tumor growth and progression.
We will review pre-clinical and clinical data in support of the concept that the cross-talk between neoplastic and immune cells is a key determinant of tumor behavior and treatment outcomes. The mediators of this cross-talk that have been identified in breast cancer will be discussed. Ultimately, improved understanding of the potential double-edge sword quality of therapies targeting mediators of this cross-talk is essential for a cautious use of immune response modifiers to harness the positive (anti-tumor immune reactivity) without promoting the negative (tumor growth, immune suppression) effects.

2. Immune cells infiltrating breast cancer

The presence of an inflammatory infiltrate in benign breast is not uncommon and may be seen in association with a variety of fibrocystic changes or conditions such as mammary

duct ectasia. However, for the most part immune cells are not a significant component of the normal breast stroma. In contrast, a marked increase in adaptive and innate immune cells often accompanies the process of carcinogenesis, with prominent inflammatory infiltrates seen around ducts involved by in situ carcinoma, as well as within invasive breast cancers (figure 1) (DeNardo and Coussens, 2007). The innate immune system plays a major role in maintenance of tissue homeostasis and reacts to tissue disruption, including physiological tissue disruption that occurs in the breast during branching morphogenesis at puberty and pregnancy, and in post-weaning involution. Macrophages, for example, have been shown to be important regulators of these processes (Gyorki and Lindeman, 2008). These physiological processes are self-limiting and the inflammation associated with them resolves once tissue homeostasis is restored. In contrast, carcinogenesis is a chronic process, often characterized by disorderly proliferation and death of the neoplastic cells, such as seen in ductal carcinoma in situ (DCIS). Deregulated cell death can foster a status of chronic inflammation, possibly due to the release of DAMPs from the dying cells (Mantovani et al., 2008; Zeh and Lotze, 2005). Death of epithelial cells that have undergone or are undergoing transformation also releases tumor-associated antigens and can result in activation of tumor-specific T and B cell responses. These immune responses can prevent tumor outgrowth, but eventually genetically unstable cancer cells give rise to variants that have become resistant to the recognition and/or killing by immune effector cells, a process defined as immunoediting (Schreiber et al., 2011). Escape from immune control does not necessarily require the loss of the antigen(s) recognized by T cells, but it is a complex process involving the production of immunosuppressive cytokines and the recruitment of regulatory innate and adaptive immune cells that protect the tumor from rejection. Key players in development and maintenance of the pro-tumorigenic environment are myeloid cells and subsets of CD4 T cells functionally differentiated towards T-helper type 2 (Th2) and regulatory (Treg) phenotypes that actively maintain a state of tolerance to the tumor (Disis, 2010). The contribution of Th2 CD4 cells has been recently demonstrated in an experimental study showing that interleukin (IL)-4 produced by Th2 CD4 T cells regulates the function of macrophages and promotes their pro-tumorigenic M2 phenotype in a mouse breast cancer model (Allavena et al., 2008; DeNardo et al., 2009). Interestingly, IL-4 has also been shown to be produced by primary epithelial breast cancer cells and to serve as an autocrine survival factor (Todaro et al., 2008). Another Th2 cytokine, IL-13, was shown to be involved in growth of human breast cancer cells (Aspord et al., 2007). Finally, a correlation between the number of Treg infiltrating human breast cancer and worse prognosis was reported in a study of 62 patients with DCIS and 237 patients with invasive breast cancer (Bates et al., 2006).

Conversely, evidence of effective anti-tumor immunity limiting tumor growth has been reported in several studies. Presence of a gene signature rich in Th1 and CD8 T cell markers was associated with a better outcome regardless of the type of epithelial malignancy in a study analyzing the stroma of primary breast cancers (Finak et al., 2008). Other studies, however, found that the prognostic value of immune signatures is different depending on the molecular subtype of breast cancer, and is a dominant factor in hormone receptors- and human epidermal growth factor receptor (Her)-2-negative (triple negative) cancers (Calabrò et al., 2009; Desmedt et al., 2008; Kreike et al., 2007; Mahmoud et al., 2011).

Overall, accumulating data support the concept that the balance between pro-tumorigenic and anti-tumor immune reactions is a key determinant of breast cancer progression. As

detailed below, the neoplastic epithelial cells both secrete and respond to cytokines, chemokines and other bioactive molecules that regulate the recruitment and function of immune cells.

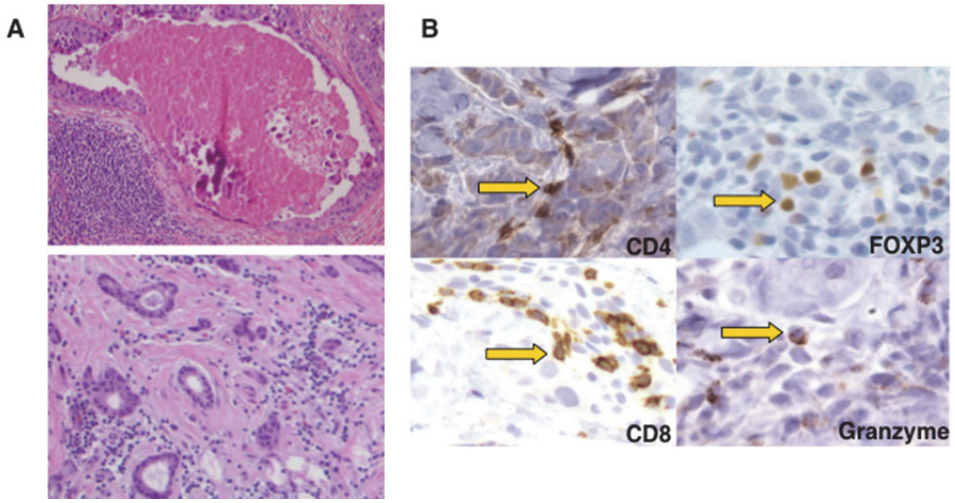

Fig. 1. Example of immune infiltrate in breast cancer. (A) Lymphocytic infiltrate as seen in H&E-stained sections. Ductal carcinoma in situ (DCIS) with comedo necrosis (upper panel), well differentiated invasive ductal carcinoma (lower panel). (B) Immunohistochemical staining of intratumoral T cells for markers of helper T cells (CD4), regulatory T cells (FoxP3), and effector T cells (CD8 and granzyme).

3. Chemokines and cytokines produced by breast cancer cells

A large network of chemokines and their receptors regulate trafficking and recruitment of innate and adaptive immune cells to different tissues in response to inflammation (Kunkel and Butcher, 2002). Signaling *via* chemokine receptors regulates processes such as cell migration, invasion, interaction with the endothelium and extracellular matrix, as well as survival. Interestingly, many epithelial cells acquire the expression of chemokine receptors and/or secrete chemokines when they undergo neoplastic transformation (Balkwill, 2004). The production of chemokines by cancer cells has been shown to influence the degree and phenotype of the inflammatory infiltrate. For example, the chemokine CCL2 (also known as monocyte chemotactic protein-1, MCP-1) is frequently secreted by breast cancer cells and is primarily responsible for recruitment of monocytes to tumors (Ueno et al., 2000; Valković et al., 1998). Within the tumor microenvironment monocytes differentiate into tumor-associated macrophages (TAM), which play a role in cancer progression and metastasis by producing immunosuppressive cytokines and pro-angiogenic factors (Pollard, 2004; Ueno et al., 2000). In human breast cancer levels of CCL2 correlate with a poor prognosis (Saji et al., 2001; Ueno et al., 2000) and recent evidence indicates that CCL2 plays a key role in pulmonary metastases of breast cancer by recruiting $Gr1^+$ inflammatory monocytes (Qian et al., 2011). Another chemokine produced by breast cancer cells and implicated in recruitment of monocytes is CCL5 (also known as Rantes) (Luboshits et al., 1999). Co-expression of CCL5

with CCL2 was reported to be associated with more advanced breast cancer stage (Soria et al., 2008).

Levels of two pro-inflammatory and pro-angiogenic chemokines, CXCL8 (also known as IL-8) and CXCL1 (also known as Growth-related oncogene, GRO) were found to be significantly elevated in sera of metastatic breast cancer patients with Her-2-positive compared with Her-2-negative cancers (Vazquez-Martin et al., 2007). *In vitro*, over-expression of Her-2 in human breast cancer cells MCF7 led to a marked increase in release of CXCL8 and CXCL1 that was abrogated by treatment with the tyrosine kinase inhibitor geftinib (Iressa), suggesting that these chemokines may play a role in the aggressive behavior of Her-2-positive breast cancers (Vazquez-Martin et al., 2007). CXCL1 and CXCL8 recruit neutrophils to tumors, and there is evidence that in the tumor microenvironment these cells acquire a pro-tumor phenotype in response to transforming growth factor (TGF)β (Fridlender et al., 2009).

Secretion of the chemokines CCL20 (also known as macrophage inflammatory protein, MIP-3α) and CCL19 (MIP-3β) by human breast cancer cells has been implicated in the recruitment of immature dendritic cells (DC) to breast cancer but the prognostic value remains uncertain (Bell et al., 1999; Treilleux et al., 2004). Interestingly, infiltration of breast cancer by Treg cells, which are recruited by CCL22 produced by approximately 60% of breast cancers (Gobert et al., 2009), was found to be associated with increased risk of relapse (Bates et al., 2006).

Conversely, some chemokines produced by breast cancer cells enhance recruitment of anti-tumor T cells. One such example is CXCL16, a chemokine that is up-regulated during inflammation in peripheral tissues and promotes recruitment of activated CD8 and Th1 T cells (Sato et al., 2005; Yamauchi et al., 2004). This may explain why the levels of expression of CXCL16 in colorectal carcinoma correlate with increased infiltration of tumors by T cells and better prognosis (Hojo et al., 2007). We were the first to report the expression of CXCL16 by human and mouse breast cancer cells, and to show that CXCL16 is markedly induced *in vitro* and *in vivo* by treatment with radiotherapy (Matsumura et al., 2008). We also showed in a mouse model of metastatic breast cancer that induction of CXCL16 by radiotherapy enhanced tumor infiltration by CD8 T cells elicited by immunotherapy promoting immune-mediated tumor rejection (Matsumura et al., 2008). Data in the preclinical model suggest that CXCL16 may play a role in response to treatment with radiotherapy and immunotherapy. Although the prognostic value of the expression of CXCL16 in breast cancer remains to be determined, it is possible that in the absence of treatment-induced anti-tumor CD8 T cells the lymphocytes recruited to $CXCL16^+$ tumors may instead promote pro-tumorigenic inflammation, as suggested in prostate cancer (Darash-Yahana et al., 2009). Whether the pro- or anti-tumor effects of CXCL16 prevail may be determined by expression of the cognate receptor, CXCR6, by the cancer cells, as discussed in the next section. Overall, chemokines expressed by breast cancer cells play critical roles in shaping the tumor immune infiltrate and likely influence tumor progression and response to treatment.

Among cytokines produced by breast cancer cells, the role of TGFβ in tumor development and progression has been extensively studied. Acting as a tumor suppressor early on, TGFβ later becomes a key factor in promoting tumor progression, metastases, and resistance to treatment (Barcellos-Hoff and Akhurst, 2009). Relevant to the focus of this chapter, in addition to direct effects on the neoplastic cells, TGFβ acts on innate and adaptive immune cells suppressing their function (Gorelik and Flavell, 2001; Wrzesinski et al., 2007). DCs

(Kobie et al., 2003) and effector CD8 T cells (Gorelik and Flavell, 2001; Thomas and J., 2005; Wrzesinski et al., 2007) are key targets of TGFβ suppressive effects in cancer leading to defects in activation and function of anti-tumor effector cells. Interestingly, an unexpected tumor-promoting effect of TGFβ was shown to be mediated by induction of production of the pro-inflammatory cytokine IL-17 by CD8 T cells (Nam et al., 2008). IL-17 acted as a survival factor for tumor cells, including mouse breast cancer cell lines that ectopically expressed the IL-17 receptor (Nam et al., 2008). These intriguing observations emphasize the complexity of interactions between tumor cells and immune system. Breast cancer cells also produce IL-4 and use it as an autocrine survival factor (Todaro et al., 2008).
The expression and production of IL-10 and IL-12 p40, but not of IL-12 p70, by human breast tumor cells was recently reported (Heckel et al., 2011). IL-10 has immunosuppressive anti-inflammatory effects, and IL-12p40 can bind to IL-12 receptor on immune cells and work as an antagonist of IL-12p70, a cytokine that promotes Th1 T cell differentiation. Although the contribution of IL-10 and IL-12p40 produced by breast cancer cells to generation of an immune suppressive tumor microenvironment remains to be further studied, data support the concept that tumors that become clinically apparent have undergone multiple changes to escape immune rejection (Schreiber et al., 2011).

4. Chemokine receptors expressed by breast cancer cells

Cancer cells express several chemokine receptors, and exploit the chemokine system to home to bone marrow and different organs that are sites of metastases. An example is CXCR4, the chemokine receptor most commonly found on cancer cells and the role of which has been more extensively characterized (Balkwill, 2004). *In vitro*, binding of CXCR4 to its ligand, the chemokine CXCL12 (also known as stromal derived factor -1, SDF-1) activates migration and invasion of cancer cells. *In vivo*, expression of CXCR4 is associated with metastatic capacity in melanoma, breast, and other cancers (Balkwill, 2004; Muller et al., 2001). Another chemokine receptor that is required for homing of lymphocytes and DCs to lymph nodes, CCR7, has been shown to be expressed by breast cancer cells and guide their metastases to lymph nodes (Muller et al., 2001).
CXCR3, a chemokine receptor expressed by activated Th1 and effector CD8 T and natural killer (NK) cells, binds to three chemokines, CXCL9, CXCL10 and CXCL11. Overexpression of CXCL10 (also known as interferon (IFN)-γ inducible protein 10, IP-10), or CXCL9 (also known as monokine induced by IFN-γ, Mig) by genetic engineering of tumor cells in experimental mouse tumor models enhanced recruitment of T and NK cells and promoted immune-mediated tumor rejection (Luster and Leder, 1993; Walser et al., 2007). However, CXCR3 is also expressed by human and mouse breast cancer cell lines (Goldberg-Bittman et al., 2004; Walser et al., 2006), and more recently it was found in all human primary breast cancers tested (N=75). Importantly, high CXCR3 expression, found in 24% of the tumors, was associated with poor overall survival (Ma et al., 2009). In experimental mouse models, blocking CXCR3 with a small molecule inhibitor prior to i.v. injection of the tumor cells, or by gene silencing in the tumor cells inhibited metastases (Ma et al., 2009; Walser et al., 2006). Intriguingly, inhibition of lung metastases by CXCR3 gene silencing required NK cells and was compromised in IFN-γ-deficient mice (Ma et al., 2009). These data highlight the complexity of the interactions between tumor and host, and caution that the systemic use of CXCR3 inhibitors could elicit mixed effects by reducing metastases while potentially interfering also with recruitment of immune cells that are required for metastasis control.

Similarly to CXCR3, CXCR6 is expressed on immune cells with anti-tumor effector function, namely activated CD8 and Th1 CD4 T cells, NK cells, and NKT cells (Kim et al., 2002; Kim et al., 2001; Nakayama et al., 2003; Unutmaz et al., 2000). CXCL16, the only ligand for CXCR6, was first shown to be expressed by immune cells with antigen-presenting function, and to be up-regulated during inflammation in different organs (Sato et al., 2005; Yamauchi et al., 2004). As mentioned above, expression of CXCL16 was recently described in several tumors, including breast cancer. Autocrine effects of CXCL16 binding to CXCR6 expressed on the same cancer cells were described in prostate cancer, where signaling via CXCR6 induced the activation of AKT/mammalian target of rapamycin (mTOR) pathway and promoted tumor cell invasion, growth and angiogenesis (Wang et al., 2008). In contrast, in renal cell carcinoma, CXCL16 expression was associated with better prognosis in patients. Endogenous CXCL16 appeared to inhibit growth and migration by interacting with CXCR6 expressed by the same tumor cells (Gutwein et al., 2009). Whether the pro- or anti-tumor effects of the CXCL16/CXCR6 pathway depend on the levels of CXCR6 expression on the tumor cells or its interaction with different forms of CXCL16 remains to be clarified. CXCL16 is one of only two chemokines that is released by cleavage of the chemokine domain from a transmembrane molecule by the activity of the disintegrin-like metalloproteinase ADAM10 (Abel et al., 2004). Soluble CXCL16 has chemotactic activity, while the transmembrane form can mediate adhesion to $CXCR6^+$ cells, as well as function as a scavenger receptor for oxidized low density lipoproteins, phosphatidylserine, and dextran sulfate (Shimaoka et al., 2003). Therefore, it is possible that interaction of CXCR6 expressed on tumor cells with the soluble chemokine domain or the transmembrane form of CXCL16 has different consequences. Expression of CXCR6 was initially reported in mouse breast cancer cell lines (Wang et al., 2006). A recent report in human breast cancer cells shows that CXCR6 can mediate chemotaxis in response to soluble CXCL16. Interestingly, expression of CXCR6 was regulated by hypoxia via hypoxia inducible factor (HIF)-1α, suggesting a role of CXCR6 expressed in breast cancer cells in cell migration in response to hypoxia (Lin et al., 2009). Although intriguing, these findings need confirmation in functional experiments assessing the role of CXCR6 in breast cancer metastasis. Overall, more data is required to clarify the expression and function of CXCR6 in breast cancer.

Another chemokine receptor, CCR5, has been implicated in breast cancer metastases promoted by mesenchymal stem cells. Intriguingly, the increased metastatic ability was dependent on the production of CCL5 by mesenchymal stem cells, which was induced de novo by the breast cancer cells, highlighting the importance of the tumor microenvironment in the cross-talk between neoplastic and stromal cells (Karnoub et al., 2007).

5. TLR and their ligands

Immune surveillance by cells of the innate immune system is mediated in large part by pattern recognition receptors (PRRs) that allow sensing of the invading pathogens and initiation of the inflammatory cascade (Kopp and Medzhitov, 2003). PRRs represent a family of evolutionarily conserved, germline-encoded proteins that recognize structural motifs found in bacteria and viruses known as pathogen-associated molecular patterns (PAMPs) (Barton and Medzhitov, 2002). TLRs constitute the most well-studied and characterized family of PRRs. To date, 11 TLRs and their cognate ligands have been identified in humans. TLRs are predominantly expressed in DCs, macrophages and NK cells. TLR activation by their respective PAMPs induces the release of pro-inflammatory cytokines, chemokines as

well as adhesion molecules that collectively enhance phagocytosis, microbial killing as well as recruitment of adaptive immunity (Iwasaki and Medzhitov, 2004).

In addition to sensing microbial pathogens, TLRs are also activated by endogenous ligands and trigger a sterile form of inflammation. First described by Matzinger as DAMP, these endogenous danger signals are often released or expressed in the context of tissue injury by both normal and neoplastic cells (Bianchi, 2007; Gallucci et al., 1999). Several recently identified DAMPs include heat- shock proteins (Ohashi et al., 2000; Roelofs et al., 2006; Vabulas et al., 2002), uric acid crystals (Liu-Bryan et al., 2005) and extracellular matrix proteins (Okamura et al., 2001) (Figure 2).

DAMP-TLR interactions have been implicated in the pathogenesis of immune dysfunction in autoimmune diseases and atherosclerosis, as well as in the chronic inflammation often associated with cancer (Marshak-Rothstein, 2006).

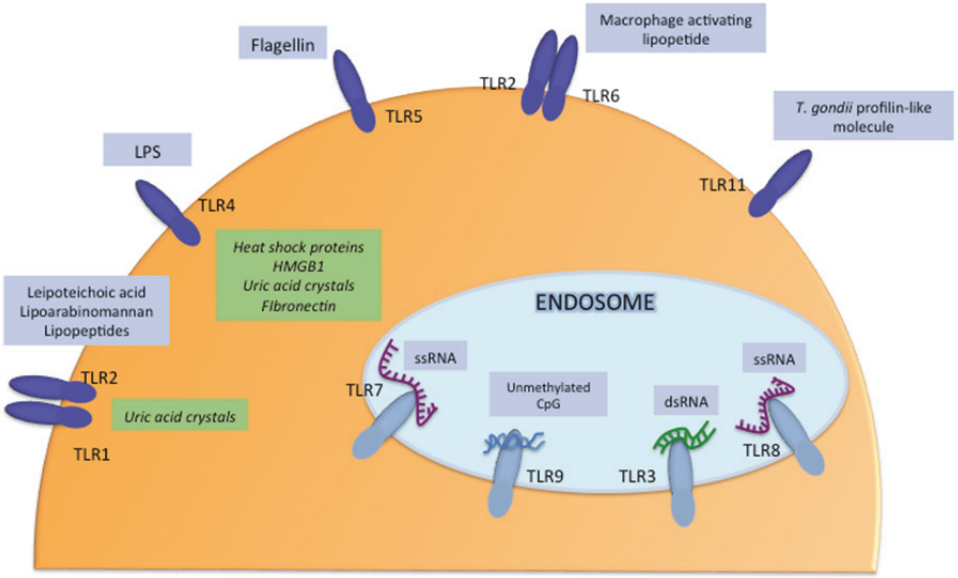

Fig. 2. Overview of toll-like receptors (TLRs) and their ligands. Activation of TLRs can be induced by exogenous microbial-derived ligands (PAMPs) as well as endogenous ligands (DAMPs) which are released from tissues in response to injury and inflammation.

Importantly, DAMP-TLR interactions have also been shown to play a decisive role in shaping anti-tumor immune responses (Apetoh et al., 2007a). Tumor cell death induced by some chemotherapy drugs and ionizing radiation resulted in release of copious amounts of the DAMP high-mobility-group box 1 (HMGB1) that binds to TLR4 expressed by DC and promotes the cross-presentation of tumor-derived antigens to T cells (Apetoh et al., 2007b). The ability of TLR engagement to activate innate immune cells to promote a defense response by inducing adaptive anti-tumor responses has spurred efforts to exploit TLR agonists as novel adjuvants for cancer therapy (Adams, 2009). Both purified natural and synthetic TLR ligands have been used in a variety of vaccination regimens designed to

overcome tolerance and sustain tumor-specific T-cell responses. Evidence from pre-clinical and clinical studies has shown the benefit of TLR stimulation when combined with conventional cancer treatment modalities such as radiotherapy and/or chemotherapy (Manegold et al., 2008; Mason et al., 2006). The discovery that many epithelial cells, including carcinoma cells, do express at least some TLRs, however, has raised the question about the effect of TLR stimulation on the tumor cells (Yu and Chen, 2008), and the effects of their therapeutic use (Huang et al., 2008). For instance, data from both mouse and human cancer cells show that while activation of some TLRs can increase susceptibility of tumor cells to apoptosis (Salaun et al., 2006), the ligation of other TLRs promotes tumorigenesis on several levels. Indeed, in a variety of tumor models, TLR stimulation has been shown to enhance proliferation, diminish tumor susceptibility to apoptosis, stimulate migratory capacity and invasiveness as well as promote angiogenesis (Harmey et al., 2002; Jego et al., 2006; Pidgeon et al., 1999). In the following section, we summarize data about the function of the main TLRs known to be expressed by breast cancer cells.

5.1 TLR3

Several TLRs (TLR 3,7,8 and 9) that recognize nucleic acid ligands are expressed intracellularly in the endosomal compartment, thus allowing for rapid detection of foreign nucleic acid material (Liu et al., 2008). TLR3 is an important detector of viral infection since it binds viral double-stranded RNA (dsRNA) and initiates a strong IFN type I response. Synthetic dsRNA agonists for TLR3, such as polyadenylic-polyuridylic acid [poly(A:U)], have been developed and tested in clinical trials in several cancers, including breast cancer, with encouraging results (Lacour et al., 1980). Interestingly, TLR3 is expressed by breast cancer cells and its triggering promotes apoptosis (Salaun et al., 2006) (Figure 3). In a recent clinical trial, adjuvant treatment with poly(A:U) showed a significant decrease in the risk of metastatic relapse in TLR3 positive but not in TLR3-negative breast cancers, suggesting that the direct anti-tumor effect may be more important than the indirect stimulation of anti-tumor immunity (Salaun et al., 2011). TLR3 triggering can also elicit the production by some tumor cells of chemokines that recruit immune cells with opposing effects (Conforti et al., 2010). Therefore, the use of TLR3 agonists should be combined with strategies to enhance anti-tumor Th1 responses and/or decrease immunosuppressive cells responsive to CCL5.

5.2 TLR4

The prototypical and best-characterized agonist for TLR4 activation is lipopolysaccharide (LPS), a structural component of Gram-negative bacteria. TLR4 can also be stimulated by viral components derived, for example, from respiratory syncytial virus (Kurt-Jones et al., 2000) or the murine retrovirus MMTV (Rassa et al., 2002). Additionally, endogenous DAMPs such as heat-shock proteins and HMGB-1 are ligands for TLR4 (Apetoh et al., 2007b; Ohashi et al., 2000). A synthetic derivative, i.e., monophospohryl lipid A (MPL), is used as a vaccine adjuvant for hepatitis B (Fendrix) and human papilloma virus (Cervarix) (reviewed in (Adams, 2009)). In the 1990's, MPL was included as a component of DETOX adjuvant in tumor vaccines for skin, lung and breast malignancies, with promising results in Phase II/III clinical trials (Eton et al., 1998; He et al., 2007; MacLean et al., 1993). On the other hand, recent evidence has implicated TLR4 expression in tumor cells as having a profound impact on tumor cell survival by evading host anti-tumor responses (He et al., 2007) or promoting

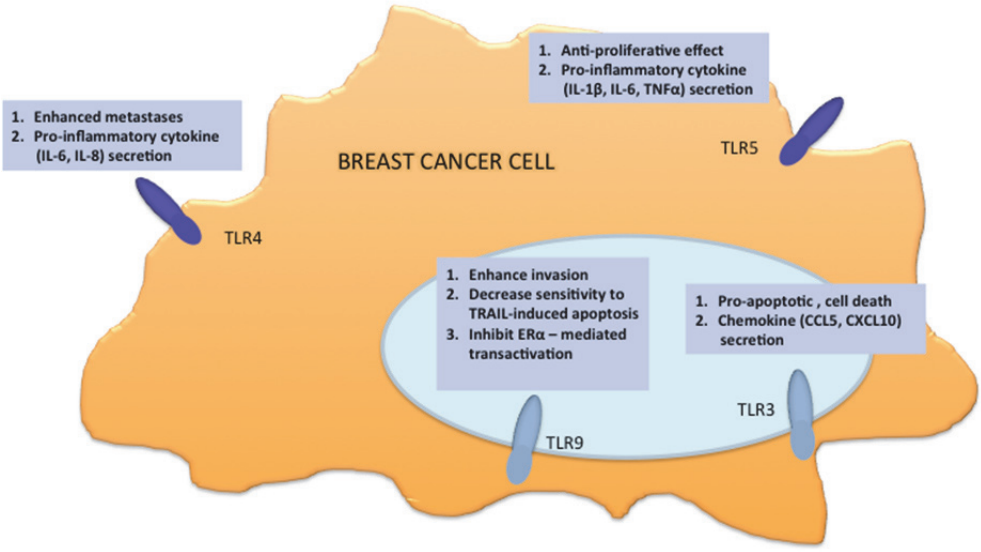

Fig. 3. Documented effects of TLR ligation on breast cancer cells. TLR activation in breast cancer cells is complex since it can either promote tumor cell death or enhance its growth and invasive potential. Like most other epithelial malignancies, breast cancer cells express several TLRs although the endogenous ligands for many of these TLRs remain unknown.

chemoresistance (Kelly et al., 2006). Expression of TLR4 by a large majority (~90%) of primary breast cancers was detected by immunohistochemistry in a study of 133 cases, but there was no significant association between TLR4-positivity and outcome (Petricevic et al., 2011). On the other hand, another study reported an interesting correlation between metastatic propensity and expression of TLR4 among stromal cells (i.e mononuclear inflammatory cells), which are found in abundance in primary breast tumors (Gonzalez-Reyes et al., 2010). These findings reiterate the complexity of the role that stromal cells play in tumor progression and suggest that TLR4 expression may be a critical mediator in these events. Furthermore, TLR4 was the predominant TLR detected in the immortalized human breast cancer cell line MDA-MB-231 (Yang et al., 2010). Knockdown of TLR4 significantly inhibited growth and secretion of IL-6 and IL-8 by these breast cancer cells, suggesting that TLR4 could be a therapeutic target. Overall, while stimulation of TLR4 in the cancer cells themselves may have deleterious effects, stimulation of TLR4 in innate immune cells could have opposite effects, depending on the tumor microenvironment, the type of myeloid cells involved (e.g., macrophages versus DC) and the availability of other signals that have to be integrated by DC to promote, rather than suppress, anti-tumor immune responses (Zitvogel et al., 2010).

5.3 TLR5

TLR5 is a cell surface receptor that recognizes bacterial flagellin and is unique among TLRs in that it is highly expressed in DCs within the lamina propria of the gut epithelium. It has also been detected in carcinomas of the gastro-intestinal tract, where it has been hypothesized that it may interact with bacterial pathogens linked to cancer development

such as Helicobacter pylori (Schmausser et al., 2005). Interestingly, a functional TLR5 is also expressed by human prostate cancer cells and its stimulation triggers the production of chemokines that recruit immune cells, although it is unclear whether recruited cells contribute to pro-tumorigenic inflammation or tumor rejection (Galli et al., 2010). On the other hand, the pro-inflammatory effects of TLR5 activation, particularly IL-6 and CCL2 release, were implicated in tumor progression of ovarian malignancies (Zhou et al., 2009). Indeed, early studies comparing TLR5 expression in normal and ovarian cancer have suggested that TLR5 could be a promising biomarker for malignant changes (Kim et al., 2008).

In a preclinical model of breast cancer, administration of flagellin to mice with established tumors inhibited the growth of an immunogenic variant expressing human Her-2 but not the parental non-immunogenic tumor (Sfondrini et al., 2006). TLR5 stimulation by flagellin was associated with enhanced IFNγ production and diminished infiltration of Treg cells. Interestingly, flagellin treatment at the time of tumor implantation had the opposite effect, leading to decreased IFNγ, increased frequency of Treg cells and accelerated tumor growth, indicating that opposing effects may be elicited depending on the tumor/host environment at time of administration (Sfondrini et al., 2006). However, since TLR5 expression in tumor cells themselves was not definitively established, no conclusions could be drawn whether the pro- or anti-tumorigenic effects of flagellin treatment resulted from direct effects on carcinoma cells.

A recent study in human primary breast cancer specimens from 75 patients demonstrated that TLR5 is expressed in normal ductal epithelium and in 80% of breast cancers examined (Cai et al., 2011). TLR5 was also expressed in 6 human breast cancer cell lines, and flagellin treatment inhibited tumor cell proliferation *in vitro* and *in vivo*, in a xenograft model. In MCF7 cells, flagellin stimulation induced tumor necrosis factor (TNF)-α, IL-1β, IL-6, and IL-8 mRNA, suggesting that flagellin activates TLR5-dependent signaling pathway in breast cancer cells. The production of several chemokines was also increased by flagellin, including MIP-3α, MCP-1, macrophage-derived chemokine (MDC), IL-6, Gro-α, and osteoprotegerin. *In vivo*, flagellin-treated MCF7 and MDA-MB-468 tumors growing in nude (T cell deficient) mice showed increased infiltration by neutrophils (Cai et al., 2011). It will be important to establish, however, if these anti-tumor effects can be achieved in immunocompetent mice.

5.4 TLR9

TLR9 is located intracellularly in the endoplasmic reticulum and binding induces translocation to the endosomal/lysosomal compartment. In humans, TLR9 is abundantly expressed in plasmacytoid DC (pDCs) and B cells. Until recently, TLR9 has been thought to recognize hypomethylated CpG deoxynucleotides (CpG-ODN) motifs characteristic of bacterial DNA but molecular studies have definitively shown that TLR9 binds instead to the 2′-deoxyribose sugar backbone (Haas et al., 2008). TLR9 activation in pDC enhances their maturation into more efficient antigen presenting cells and producers of powerful pro-inflammatory cytokines such as type I IFN (Gilliet et al., 2008). Furthermore, the activation of TLR9 in B cells promotes their proliferation and polyclonal immunoglobulin synthesis, thus generating a robust humoral response as well (Chiron et al., 2008). The broad spectrum of immunoactivating effects of TLR9 stimulation on both innate and adaptive responses have spurred efforts to use synthetic TLR9 ligands as an immunotherapeutic for both solid

tumors and hematological malignancies (Krieg, 2008). Initially, TLR9 expression was thought to be restricted to immune cells, but recent studies have conclusively showed that a variety of tumor cell types also express functional TLR9 molecules. Indeed, expression of TLR9 has been confirmed in both frozen breast tumor specimens (Berger et al., 2010)as well as breast cancer cell lines (Berger et al., 2010; Merrell et al., 2006; Qiu et al., 2009). A study of 124 frozen breast tissue specimen from women diagnosed with breast cancer found a positive correlation (Spearman rank p=0.04) between TLR9 mRNA expression and increasing tumor grade, suggesting that TLR9 expression may be a molecular marker for poorly differentiated breast cancers (Berger et al., 2010).

The direct effects of TLR9 stimulation on tumor cells, however, remains decidedly complex. In 2006, Selander and colleagues showed that CpG-ODN stimulation of the TLR9-positive MDA-MB-231 but not TLR9-negative MCF-7 human breast cancer cells induced their migration across a matrigel matrix (Merrell et al., 2006), suggesting that TLR9 signaling plays a role in cancer progression and metastasis. TLR9 overexpression in BT-20 breast cancer cells has similarly been found to enhance invasiveness *in vitro* (Berger et al., 2010). In both studies, CpG-ODN stimulation did not affect cellular proliferation, thus negating the possibility that the enhanced migration could be attributed to increased cell division. TLR9 expression may also be a mechanism that tumors employ to evade host immune responses such as tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-induced apoptosis. The TRAIL/TRAIL receptor interaction is an important mechanism by which anti-tumor effectors such as CD8 T cells, NK cells and NKT cells mediate tumor-directed cell kill. In an *in vitro* study using TLR9-expressing breast cancer cell lines HCC1569 and Cal51, CpG-ODN stimulation resulted in a significant decrease in the sensitivity of tumor cells to lexatumumab, an anti-DR5 agonist antibody that stimulates the TRAIL pathway (Chiron et al., 2009; Ohta et al., 2006) . Using a synthetic TLR9 ligand in which the phosphate backbone was modified to increase resistance to nucleases and enhance circulating half-life, Chiron and co-workers showed that the phosphorothioate-modified TLR9 agonist could bind directly to the DR5 receptor on tumor cells and inhibit TRAIL-dependent killing by NK cells. These findings have important implications for the use of TLR9-directed therapies using synthetic CpG-ODNs which may potentially attenuate tumor immunosurveillance. Conversely, a recent study suggest that CpG-ODN stimulation may hold therapeutic promise in estrogen-responsive breast cancer cells (Qiu et al., 2009). TLR9 activation in T47D and MCF-7 breast cancer cells inhibited estrogen-receptor alpha (ERα)-mediated transactivation through the NF-κB pathway. Although these findings need to be confirmed in primary breast tumor tissues, it is intriguing to investigate whether CpG-ODN stimulation can synergize with hormonal therapy for ER^+ breast cancers.

6. The cross-talk between regulatory T cells and breast cancer cells (RANKL)

Receptor activator of NF-kB (RANK) is a type I membrane protein, which shares high homology with CD40. RANK ligand (RANKL, also called TRANCE (TNF-related activation-induced cytokine) or osteoclast differentiation factor (ODF) is a type II membrane protein with belongs to the TNF superfamily originally identified as a dendritic cell survival factor. RANKL is predominately expressed in activated T cells, as well as the thymus, lymph node and bone marrow. RANK/RANKL are essential regulators of bone remodeling, body temperature, lymph node and thymus formation as well as mammary gland development

during pregnancy (Leibbrandt and Penninger, 2008). Furthermore, the RANK/RANKL axis has been linked to progestin-driven breast carcinomas and bone metastases (Schramek et al., 2010).

In addition to the expression of RANK on hematopoietic osteoclast precursors and DC, the receptor is also expressed by some tumor cell types, including melanoma, osteosarcomas, breast and prostate cancers (Jones et al., 2006; Mori et al., 2007a; Mori et al., 2007b). RANK expression has been reported in 6-57% of invasive human breast cancers, depending upon the parameters used to define positivity and antibodies utilized for staining (Gonzalez-Suarez et al., 2010; Santini et al., 2011). Stimulation of RANK+ human breast cancer cells with recombinant RANKL induces actin polymerization and migration without affecting cell proliferation (Jones et al., 2006). Preclinical models of Her-2+ mammary carcinoma (MMTV-neu transgenic mouse) have shown that metastatic spread is dependent on RANK signaling and that pharmacological inhibition of RANKL reduces tumor growth and lung metastases (Gonzalez-Suarez et al., 2010; Tan et al., 2011).

While only a subgroup of breast cancers expresses RANKL and there is no evidence for co-localization of RANK and its ligand in the carcinoma epithelium (Gonzalez-Suarez et al., 2010; Van Poznak et al., 2006), RANKL is expressed by infiltrating immune cells. In one study, RANKL was detected in tumor-infiltrating mononuclear cells (not further characterized) and occasionally in fibroblast-like stromal cells (Gonzalez-Suarez et al., 2010). Another report showed that the majority of RANKL-producing cells infiltrating breast cancers were T cells expressing FOXP3, a transcription factor produced by Treg cells (Tan et al., 2011). Importantly, RANK signaling mediated the metastatic behavior of RANK-expressing mouse breast cancer cells, and RANKL was produced by Treg cells (Tan et al., 2011). Therefore, in addition to suppressing anti-tumor immune responses, Treg cells might promote the metastatic behaviors of some tumors by producing RANKL, explaining why Treg cells have been shown to have prognostic significance in breast cancers. In 237 patients with operable breast cancers, Treg cell numbers in the primary tumor correlated with relapse-free survival independently of nodal involvement, tumor size and grade (Bates et al., 2006). Therefore, it will be of great interest to determine if tumor infiltration by Treg cells and/or Th17 cells, another T cell subset that has been shown to express high levels of RANKL (Sato et al., 2006), predicts for increased metastases of RANK+ breast cancers, and whether RANKL inhibition will be effective at inhibiting metastasis and risk of recurrence and death from breast cancer.

7. Therapeutic implications

The role of interactions between tumor cells and host immune system is increasingly appreciated as critical for tumor development and progression, as well as therapeutic response. As discussed above, the type and density of immune cells infiltrating breast cancers is associated with prognosis, with high density of macrophages forecasting a worse outcome (Bingle et al., 2002) while high numbers of $CD8^+$ T cells predict a better outcome (Mahmoud et al., 2011). Importantly, the presence of a brisk lymphocytic infiltrate in pre-treatment biopsies of more than one thousand primary breast cancers was significantly associated with pathological complete response (pCR) to neoadjuvant anthracycline/taxane treatment (Denkert et al., 2010). A significant association was found between markers of T cells (CD3) and effector T-cell recruitment (CXCL9) and pCR (Denkert et al., 2010). These data in patients support the concept that the anti-cancer immune response is essential for

therapeutic success (Zitvogel et al., 2008), and suggest that immune infiltrates can provide predictive information. Indeed, if cytocidal treatments work, in part, by causing an immunogenic tumor cell death and generating an *in situ* vaccine, the presence of a less immunosuppressive microenvironment will favor development of anti-tumor immunity post-treatment (Apetoh et al., 2007c; Formenti and Demaria, 2009; Ghiringhelli et al., 2009; Obeid et al., 2007). Conversely, immune cells and their receptors become attractive targets for improving response to chemo- and radio-therapy. For example, we have shown in a mouse model of metastatic breast cancer that targeting the co-inhibitory receptor CTLA-4 on T cells synergizes with local radiotherapy in inducing the immune-mediated regression of the irradiated tumor and metastases outside of the radiation field (Demaria et al., 2005). In a different mouse model of breast cancer targeting colony stimulating factor (CSF)-1 receptor with an antagonist blocked macrophage recruitment to paclitaxel-treated tumors leading to improved therapeutic response, longer survival and reduced metastases (DeNardo et al., 2011).

Strategies to deplete Treg cells in breast cancer patients (Dannull et al., 2005; Rech and Vonderheide, 2009) may also be beneficial by reducing local immunosuppression as well as removing a main source of RANKL production. Increased accumulation of Treg cells is also seen in sentinel lymph nodes of breast cancer patients and it correlates with the size of the primary tumor (Gupta et al., 2011). Since anti-tumor T cells are activated in sentinel lymph nodes (Kim et al., 2006) the increased Treg cell presence might limit the efficacy of pre-operative chemotherapy for locally advanced breast cancer by inhibiting the activation of tumor-specific T cells (Boissonnas et al., 2010).

Multiple additional strategies for manipulating the immune environment of breast cancer are being studied, including TLR agonists (Lu et al., 2010), immunomodulatory drugs and vaccines (Emens et al., 2009). A critical question that will need to be addressed is how we predict response to treatment with agents that target the immune system, whether directly such as antibodies against co-stimulatory or co-inhibitory T cell receptors, or indirectly such as chemotherapy drugs that induce an immunogenic cell death. In fact, polymorphisms of TLR4 and P2X7, receptors that play a key role in development of anti-tumor immunity following chemotherapy-induced immunogenic tumor cell death, are present in the population and have been shown to impact response to treatment with anthracyclines and radiotherapy (Apetoh et al., 2007c; Ghiringhelli et al., 2009). Therefore, as recently proposed by Zitvogel and colleagues (Zitvogel et al., 2011), immune-relevant biomarkers will need to be considered together with tumor cell biomarkers in tailoring treatment for patients towards a personalized therapeutic approach.

8. Conclusions

This chapter summarizes the recent advances in our understanding of the interplay between breast cancer and the immune system. Cancer cells secrete and respond to cytokines, chemokines, and DAMPs influencing the nature and quantity of the immune infiltrate. In turn, the type of immune cells present within breast cancer can have a major impact on tumor progression, prognosis and response to treatment. Immune cells can foster a pro-tumorigenic inflammatory environment as well as inhibit tumors (Figure 4). To achieve therapeutic success, any treatment strategy will need to include an approach to shift the balance of pro-tumorigenic and anti-tumor immunity in favor of the latter. The good news is that enlisting the power of the immune system to synergize with cytocidal tumor therapy

holds the promise to revolutionize treatment and the hope to achieve long-term tumor control and perhaps cure (Schreiber et al., 2011).

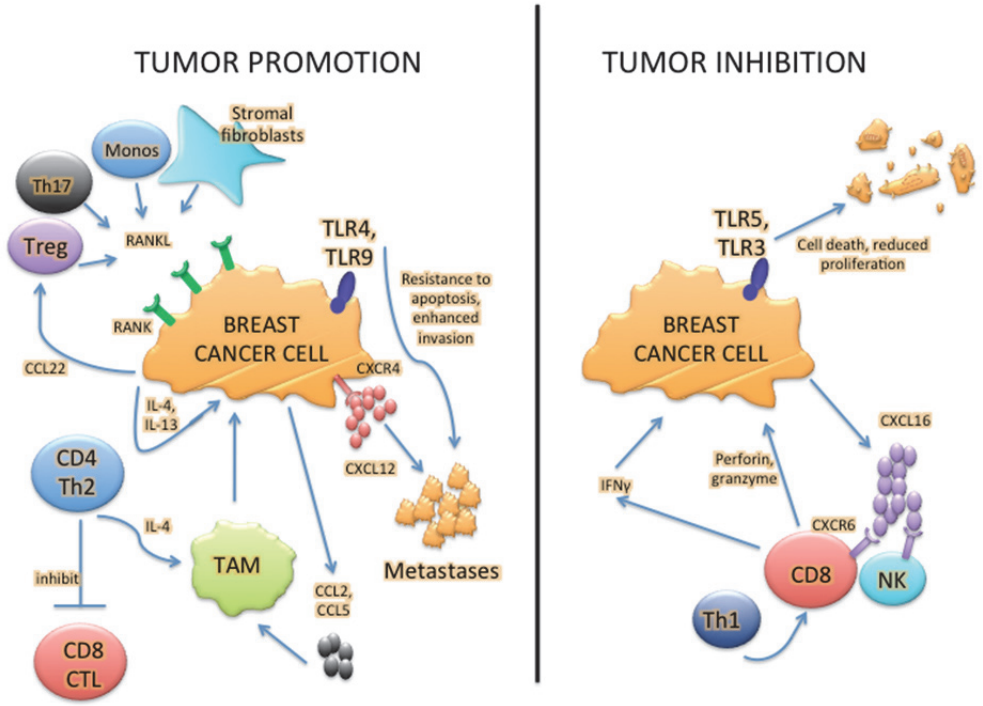

Fig. 4. Immune cells infiltrating breast cancer play a dual role, promoting (left) or inhibiting (right) tumor growth and metastases. Breast cancer cells produce chemokines, such as CCL5 and MCP-1, that recruit monocytic cells which, in the presence of IL-4 secreted by Th2 T cells differentiate into pro-tumorigenic macrophages (TAMs). Breast cancer cells also express chemokine receptors, such as CXCR4, that promote their migration in response to CXCL12, guiding metastases to distant organs. In contrast, other chemokines produced by breast cancer cells, such as CXCL16, promote the recruitment of CXCR6+ anti-tumor CD8 T cells. Activation of TLRs on the surface of breast cancer cells has differential effects that can either promote or inhibit tumor growth. The recruitment of Treg cells by breast tumor cells via secretion of CCL22 contributes to create an immunosuppressive milieu. In addition, RANKL production by Treg and Th17 cells, and possibly other stromal cells, promotes metastases of RANK+ breast cancer cells.

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Appendix 2:

Fanny Bouquet, Anupama Pal, **Karsten A. Pilones**, Sandra Demaria, Byron Hann, Rosemary J. Akhurst,Jim S. Babb, Scott M. Lonning, J. Keith DeWyngaert, Silvia C. Formenti, and Mary Helen Barcellos-Hoff. TGFb1 Inhibition Increases the Radiosensitivity of Breast Cancer Cells In Vitro and Promotes Tumor Control by Radiation In Vivo. *Clin Cancer Res*; 17(21); 6754–65.

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Clinical Cancer Research

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Cancer Therapy: Preclinical

Clinical Cancer Research

TGFβ1 Inhibition Increases the Radiosensitivity of Breast Cancer Cells *In Vitro* and Promotes Tumor Control by Radiation *In Vivo*

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Abstract

Purpose: To determine whether inhibition of TGFβ signaling prior to irradiation sensitizes human and murine cancer cells *in vitro* and *in vivo*.

Experimental Design: TGFβ-mediated growth and Smad phosphorylation of MCF7, Hs578T, MDA-MB-231, and T47D human breast cancer cell lines were examined and correlated with clonogenic survival following graded radiation doses with and without pretreatment with LY364947, a small molecule inhibitor of the TGFβ type I receptor kinase. The DNA damage response was assessed in irradiated MDA-MB-231 cells pretreated with LY364947 *in vitro* and LY2109761, a pharmacokinetically stable inhibitor of TGFβ signaling, *in vivo*. The *in vitro* response of a syngeneic murine tumor, 4T1, was tested using a TGFβ neutralizing antibody, 1D11, with single or fractionated radiation doses *in vivo*.

Results: Human breast cancer cell lines pretreated with TGFβ small molecule inhibitor were radiosensitized, irrespective of sensitivity to TGFβ growth inhibition. Consistent with increased clonogenic cell death, radiation-induced phosphorylation of H2AX and p53 was significantly reduced in MDA-MB-231 triple-negative breast cancer cells when pretreated *in vitro* or *in vivo* with a TGFβ type I receptor kinase inhibitor. Moreover, TGFβ neutralizing antibodies increased radiation sensitivity, blocked γH2AX foci formation, and significantly increased tumor growth delay in 4T1 murine mammary tumors in response to single and fractionated radiation exposures.

Conclusion: These results show that TGFβ inhibition prior to radiation attenuated DNA damage responses, increased clonogenic cell death, and promoted tumor growth delay, and thus may be an effective adjunct in cancer radiotherapy. *Clin Cancer Res; 17(21); 6754–65. ©2011 AACR.*

Introduction

Ionizing radiation is an effective cancer treatment modality that is administered in a manner that maximizes tumor damage while minimizing effects on normal adjacent tissue (1). Further benefit can be achieved through administration of drugs or biological agents that either promote tumor cytotoxicity or protect normal tissue from dose-limiting toxicity, such as fibrosis. TGFβ is a candidate target whose inhibition could potentially do both.

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There is substantial evidence that TGFβ plays a crucial role in the response to ionizing radiation (2). TGFβ is a pleiotropic cytokine that is important in normal tissue homeostasis, regulates inflammation and immune responses, and suppresses epithelial proliferation. TGFβ is activated in irradiated tissues, presumably because the latent TGFβ complex has a specific redox-sensitive conformation activated by reactive oxygen species, which are generated by radiation (3). Some preclinical models suggest that radiation-induced TGFβ contribute to metastasis (4); consistent with this irradiated cells are primed to undergo TGFβ-mediated epithelial-mesenchymal transition that increases motility and invasion (5, 6). Radiation-induced TGFβ activity can also be sustained beyond an acute response, which may drive function-compromising fibrosis, a common sequel following radiotherapy, in susceptible tissues (7–11). Significant experimental support for a critical role of TGFβ in radiation-induced fibrosis is provided by studies in which blocking TGFβ production or signaling significantly reduces fibrosis in preclinical rodent models (8, 9, 12–15). This has led to recognition that TGFβ inhibition following radiotherapy could prevent normal tissue toxicity due to fibrosis (9, 11, 16), although this concept awaits testing in clinical trials.

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Translational Relevance

Radiation therapy is an important treatment modality for breast and other cancers. We have shown that ionizing radiation induces TGFβ activation and that TGFβ inhibition compromises canonical radiation responses in epithelial cells via inhibition of ataxia telangiectasia mutated kinase activity. We hypothesized that inhibiting TGFβ during radiotherapy could increase tumor response to radiation therapy if cancer cells maintain TGFβ regulation of the DNA damage response. We show that human and mouse breast cancer cell lines were radiosensitized when pretreated with either a small molecule inhibitor of the TGFβ type I receptor kinase or pan-specific TGFβ neutralizing antibody. Consistent with this, phosphorylation of γH2AX and other DNA damage responses were significantly reduced by TGFβ inhibition prior to *in vitro* or *in vivo* irradiation. Moreover, tumor growth delay following radiation was significantly greater when TGFβ neutralizing antibodies were administered before either single or multiple radiation fractions. These preclinical studies suggest that TGFβ inhibition during radiotherapy could provide significant benefit.

A new role for TGFβ in mediating the rapid execution of the DNA damage response (DDR) has been identified (reviewed in ref. 2). Perhaps the first indication of its critical role was the observation that epithelial tissues of *Tgfb1* heterozygote embryos exposed *in utero* to 5 Gy showed significantly less radiation-induced apoptosis and *Tgfb1* null embryos fail to undergo either apoptosis or inhibition of cell cycle (17). TGFβ depletion by gene knockout or transient depletion by TGFβ neutralizing antibody also reduced radiation-induced p53 phosphorylation *in vivo* (17). Subsequent studies by Boothman and colleagues showed that TGFβ initiates a radiation survival mechanism dependent on secretory clusterin (18). However, the failure of the proximal DDR is attributed to compromised ataxia telangiectasia mutated (ATM) protein auto-phosphorylation and kinase activity, which decreases phosphorylation of critical DNA damage transducers γH2AX, Chk2, p53, and Rad17 and in turn, abrogates cell fate decisions (19). As a consequence, both *Tgfb1* null murine epithelial cells and human cells in which TGFβ signaling is pharmaceutically blocked are more radiosensitive, as measured by clonogenic survival (19). Furthermore, Rodemann and colleagues showed that TGFβ1 antisense also compromises ATM kinase–dependent phosphorylations in irradiated A549 lung cancer cells (20). ATM is a nuclear sensor of DNA damage that initiates, recruits, and activates a complex program of checkpoints for cell cycle, apoptosis, and genomic integrity and its loss or inhibition enhances radiosensitivity (see reviews in refs. 21, 22). As a consequence, radiosensitivity, as measured by clonogenic survival, increases in both murine epithelial cells from *Tgfb1* null mice and in human epithelial cells in which TGFβ signaling is pharmaceutically blocked (19).

Most solid cancers escape TGFβ growth regulation and amplify TGFβ production, which in turn suppresses immunosurveillance and enhances invasion and metastasis (23), thus leading to a protumorigenic environment. TGFβ also compromises responses to chemotherapy (reviewed in ref. 24), by mechanism that remains undefined. Taken together, increased levels of TGFβ protein in cancer, TGFβ activation by radiation, and TGFβ regulation of ATM kinase activity and DDR, suggest that TGFβ could protect cancer cells from DNA damage, thus decreasing the efficacy of radiotherapy. A small molecule inhibitor of TGFβ signaling has shown efficacy in combination with radiation and chemotherapy in human glioblastoma xenografts (25). However, breast cancer often evades the growth-inhibitory action of TGFβ by selectively eliminating cytostatic gene responses due to attenuated C/EBPβ transcriptional control (26). Although TGFβ control of ATM is not dependent on cell-cycle status per se in normal cells (19), it is unknown whether TGFβ inhibition can increase radiosensitivity when growth regulation is truncated in breast cancer cells. Thus, to assess the therapeutic potential of TGFβ inhibition in radiotherapy for breast cancer, we determined the relationship between sensitivity to TGFβ-mediated growth inhibition, molecular responses to radiation, and radiosensitivity in human and murine breast cancer cells. This study shows that TGFβ inhibition increases radiosensitivity of breast cancer cells irrespective of growth sensitivity, compromises DDR, and promotes radiation-induced tumor growth delay.

Materials and Methods

Cell culture

Human breast cancer cell lines MCF10A, MCF7, MDA-MB-231, T47D and Hs578T cells, and murine breast cancer cell line 4T1 were obtained from American Type Culture Collection and were cultured in MEGM, 10% FBS-MEME, 10% FBS-DMEM, 10% FBS-RPMI, 10% FBS-DMEM, and 10% FBS-DMEM media, respectively, at 37°C with 5% CO_2. Cells were treated in 10% serum replacement medium (SRM; Knockout SR, Life Technologies, Inc.) containing either 500 pg/mL TGFβ (R&D Systems), 400 nmol/L small molecule inhibitor of the TGFβ type I receptor kinase, LY364947 ([3-(Pyridin-2-yl)-4-(4-quinonyl)]-1H-pyrazole); Lilly designation HTS466284; Catalogue no. 616451, Calbiochem) or 1D11, a pan-isoform, neutralizing TGFβ monoclonal antibody or 13C4, murine monoclonal isotype control antibody (Genzyme). For growth studies, cells were trypsinized and counted using a Coulter counter at 24-hour and 48-hour posttreatment. Cells were grown in complete media for 48 hours, followed by LY364947 treatment (400 nmol/L) in 10% SRM for 24 hours prior to irradiation with 2 Gy for γH2AX foci induction by immunofluorescence and 5 Gy for DDR using immunoblotting.

Colony assay

Human breast cancer cells grown for 48 hours to 70% confluence were treated with 400 nmol/L of LY364947

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kinase inhibitor for 48 hours before and 3-hour postradiation exposure with differential doses using a 250-kVp X-ray (0.61 Gy/min). Murine mammary 4T1 tumor cells were treated with 1D11 or 13C4 for 24 hours before irradiation to the indicated dose with Clinac 2300 C/D linear accelerator. Cells were trypsinized 3-hour postirradiation and were plated in triplicates at 3 dilutions into 6-well cell culture plates in serum containing media. Cells were allowed to grow for 10 to 12 days followed by fixing and staining with crystal violet. Colonies containing more than 50 cells were counted. To determine percent survival, colony forming efficiency was determined, averaged, and normalized to those of the nonirradiated control. For each radiation dose, the mean number of colonies obtained from 3 wells were corrected according to plating efficiency and used to calculate the cell survival at each dose (27). The significance of the difference between the dose responses was calculated by conducting a 1-way ANOVA test. We chose the hierarchical ANOVA models to control the heterogeneity of the data caused by the interaction of the 2 variables and to infer the statistical significant differences in the clonogenic survival between treatments for the given radiation doses using factorial ANOVAs followed by least significant difference 2-by-2 comparisons.

Immunoblot analysis

To examine molecular responses to TGFβ or radiation, 7×10^5 cells were grown in complete media for 48 hour, followed by LY364947 treatment (400 nmol/L) in 10% SRM for 24 hours followed by exposure to 5 Gy, which were lysed after 1 hour, or treated with 500 pg/mL TGFβ, which were lysed after 30 minutes. The extracts were subjected to immunoblot analysis with one of the following primary antibodies: Smad2 serine 465/467 phosphorylation at 1:500 (clone 138D4, Catalogue no. 3108, Cell Signaling), Smad2/3 at 1:500 (Catalogue no. 610842, BD Transduction Laboratories), p53 serine 15 phosphorylation at 1:500 (Catalogue no. 92845, Cell Signaling), p53 serine 20 phosphorylation at 1:500 (Catalogue no. 92879, Cell Signaling), and p53 at 1:500 (Clone DO-7 + BP53-12, Catalogue no. MS-738-P0 Neomarkers). Protein estimation was carried out using the BCA protein assay kit from Pierce. One hundred micrograms of protein was electrophoresed on a 4% to 15% gradient gel from BioRad and transblotted on polyvinylidene difluoride membrane. The membrane was incubated in blocking buffer and probed with one of the primary antibodies, washed 3 times for 10 minutes with 0.1% TBST, followed by incubation with secondary antibodies (goat anti-mouse, Catalogue no. 926-32220 and goat anti-rabbit, Catalogue no. 926-32211, Odyssey) for 1 hour at room temperature. The membrane was washed 3 times for 10 minutes with TBST 0.1% and scanned on the Odyssey LICOR system.

γH2AX foci

Tumor cryosections or cells grown on chamber slides were fixed using 2% paraformaldehyde for 20 minutes at room temperature followed by permeabilization with 100% methanol for 20 minutes at −20°C. Then specimens were blocked with the supernatant of 0.5% casein/PBS, stirred for 1 hour, and incubated with a mouse monoclonal antibody against γH2AX (clone JBW301, Upstate Biotechnology) overnight at 4°C followed by washes and incubation with Alexa-486 labeled anti-mouse secondary antibodies (Molecular Probes) for 1 hour at room temperature. Specimens were counterstained with 4′,6-diamidino-2-phenylindole (DAPI), and washed in PBS-Tween20 0.1% before mounting with Vectashield mounting medium (Vector Labs). Specimens were imaged using a 40× objective with 0.95 numerical aperture Zeiss Plan-Apochromat objective on a Zeiss Axiovert (Zeiss) equipped with epifluorescence. All images were acquired with a CCD Hamamatsu Photonics monochrome camera at 1,392 × 1,040 pixel size, 12 bits per pixel depth using the Metamorph imaging platform (Molecular Devices, Inc.).

In vivo tumor studies

All animal studies were conducted using protocols that had undergone appropriate review and approval at each institution. Severe combined immunodeficient mice/beige mice were injected subcutaneously at the UCSF Preclinical Therapeutics Core with 2×10^6 MDA-MB-231 breast cancer cells on each flank at the UCSF Preclinical Therapeutics Core. LY2109761 (100 mg/kg, Eli Lilly & Co.; ref. 28) or vehicle control was administered once by oral gavage at day 29 when tumors were approximately 0.8 cm in diameter. Two hours later, the mice were irradiated or mock irradiated using a medial exposure body shield with 3 Gy Cs-radiation source. Tumors ($n = 3$ per treatment) were excised 1 hour after radiation exposure and frozen in OCT using dry ice/ethanol bath.

Balb/C mice were injected subcutaneously with 5×10^4 4T1 cells on left flank of the mice at NYU Medical Center and were treated at day 13, upon reaching approximately 100 mm^3. Neutralizing antibody 1D11 or isotype control, 13C4 (29), were administered by intraperitoneally (i.p.) injection (5 or 50 mg/kg, kindly provided by Genzyme) 24 hours before tumors were locally irradiated with a single dose of 8 Gy or 3 doses of 12 Gy on 3 consecutive days. Briefly, mice were lightly anesthetized by i.p. injection of Avertin (240 mg/kg) and then positioned on a dedicated plexiglass tray to irradiate a field including the tumor with 5-mm margins using a Clinac 2300 C/D linear accelerator (Varian Medical Systems) fitted with a 25-mm radiosurgery conical collimator (BrainLAB AG). Superflab bolus (1.5 cm tissue equivalent material) was placed over the tumor, and a source-to-skin distance of 100 cm was set. Radiation was delivered at 600 cGy/min with 6 MV X-rays. Mice were monitored thrice weekly for signs of toxicity and tumors volumes were measured with a caliper. Tumors volumes were calculated as length × $width^2$ × 0.52.

Statistical analysis

Random coefficients regression was used to assess the effect of treatment on log tumor growth; log volumes were used in place of observed volumes because the change in log volume over the course of follow-up was well

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approximated as linear. The model to predict log tumor volume included elapsed time from baseline (defined as first day of measurement) as a numeric factor, treatment as a classification factor and the term representing the interaction of treatment with time. The interaction term was partitioned to derive tests that compared treatment arms in terms of tumor growth rate. The correlation structure imparted by the inclusion of multiple-dependent variable observations per animal was modeled by assuming observations to be correlated only when acquired from the same animal with the strength of correlation between observations inversely dependent on the elapsed time between observations (i.e., measures are more strongly correlated when taken closer together in time).

After conducting a Shapiro–Wilks test to verify the Gaussian distribution of the data, an ANOVA was used to compare treatment arms in terms of the normalized tumor volumes or tumor weight observed at each day of measurement. The error variance was allowed to differ across treatment arms to avoid the unnecessary assumption of variance homogeneity. All reported *P* values are 2-sided and were declared significant at the 5% level. All computations were carried out using commercially available software (SAS 9.0; SAS Institute).

Results

Human breast cancer cell responses to TGFβ

TGFβ regulation of ATM kinase activity and downstream phosphorylation targets does not depend on cycling status in nonmalignant MCF10A cells (19), but these cells are TGFβ responsive, whereas most breast cancers proliferate in the presence of active TGFβ signaling (30). Thus, we first established the relative TGFβ growth inhibition of MCF7, MDA-MB-231, Hs578T, and T47D breast cancer cell lines in comparison with MCF10A as a positive control (Fig. 1A). Among the breast cancer cell lines, only Hs578T cells were growth inhibited by TGFβ, similar to that observed in nonmalignant MCF10A cells. MCF7 and MDA-MB-231 cells were refractory to TGFβ-mediated growth regulation while T47D cells were slightly stimulated by addition of TGFβ.

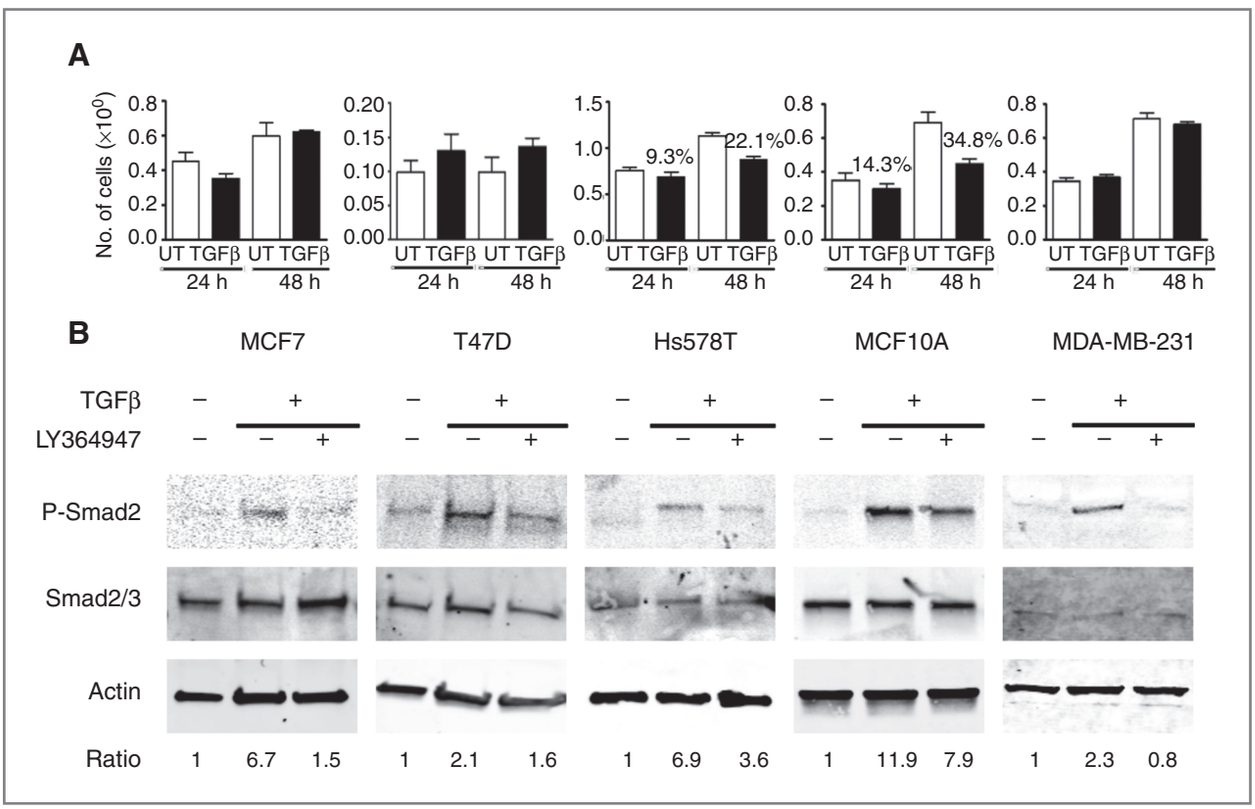

Figure 1. TGFβ growth regulation and signaling in breast epithelial cell lines. A, the bar graphs show the growth response at 24 hours or 48 hours of TGFβ treatment in 5 breast epithelial cell lines. The untreated controls are designated as "UT" and "TGFβ" refers to TGFβ-treated cells. MCF10A cells show 14% ($P = 0.38$) and 35% ($P = 0.02$) growth inhibition at 24 hours and 48 hours of TGFβ treatment. Hs578T were growth inhibited by 9% ($P = 0.36$) at 24 hours and 22% ($P = 0.004$) at 48 hours of TGFβ treatment. Proliferation of MDA-MB-231 and MCF7 cells was unaffected by TGFβ whereas proliferation of T47D cells increased slightly. B, immunoblots of phospho-Smad2, total Smad2/3 and Actin from MCF7, T47D, Hs578t, MCF10A, and MDA-MB-231 cells treated with LY364947 for 48 hours followed by TGFβ for 30 minutes or sham treated. TGFβ treatment induced phosphorylation of Smad2, which was blocked by LY364947 pretreatment. These data indicate that TGFβ signaling through the type I receptor kinase is functional and LY364947 is effective in blocking the canonical pathway of TGFβ through TGFβRI. Quantifications of the ratios of phosphorylated protein/total protein normalized to untreated control are indicated below each lane.

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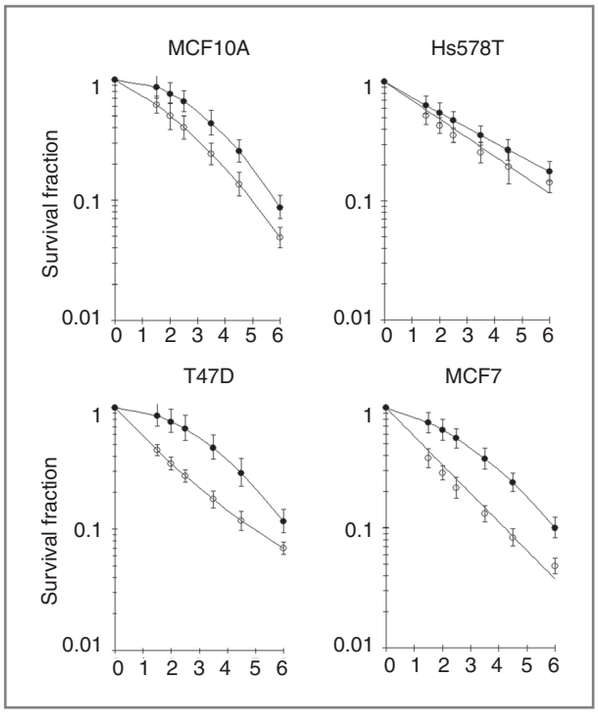

Figure 2. TGFβ inhibition by small molecule inhibitor LY364947 radiosensitizes epithelial breast cell lines. Cells treated for 48 hours with 400 nmol/L of LY364947 prior to exposure to graded doses of radiation were plated 3-hour postirradiation for clonogenic survival assay. Closed circles (●) represent untreated irradiated controls whereas open circles (○) represent the colony forming efficiency of LY364947 treated, irradiated cells. Mean ± SD values of triplicate determinations are shown. LY364947 treatment significantly increases radiation sensitivity (ANOVA Tukey test): MCF10A, $P = 0.013$, Hs578T, $P = 0.005$, T47D, $P = 0.01$, and MCF7, $P = 0.01$.

Because breast cancer cell lines can selectively evade TGFβ growth regulation while maintaining signaling (26), we next examined activation of TGFβ canonical pathway as evidenced by Smad2 phosphorylation. Extracts from cells treated for 30 minutes with TGFβ showed a 2 to 12-fold increase in Smad2 phosphorylation, which was inhibited by pretreatment with LY364947, inhibitor of TGFβ receptor I (Fig. 1B). Thus all breast cancer cell lines, irrespective of their sensitivity to TGFβ-mediated growth inhibition, showed type I receptor kinase response to TGFβ treatment.

Inhibition of TGFβ signaling increases radiosensitivity

We then determined clonogenic survival as a measure of the radiation sensitivity of these human breast cancer cell lines and evaluated the effect of TGFβ signaling inhibition prior to irradiation. As previously reported (19), the radiation sensitivity of the TGFβ responsive, nonmalignant cell line MCF10A was significantly increased when subjected to inhibition of TGFβ signaling by TGFβ type I receptor kinase small molecule inhibitor LY364947 (Fig. 2). Moreover, the radiation sensitivity of TGFβ refractory cancer cell lines, MCF7 and T47D, and TGFβ growth sensitive cancer cell line, Hs578T, was increased following TGFβ inhibition (Fig. 2). The dose enhancement ratios at 10% survival (Table 1) varied between cell lines. Although representing a small sample of breast cancer cell lines, we found no apparent association between cancer cell subtype, estrogen receptor positivity or p53 mutation status, TGFβ growth sensitivity, and the effect of LY364947 pretreatment on radiosensitization.

Inhibition of TGFβ signaling attenuates DDR in MDA-MB-231 cells *in vitro* and *in vivo*

The MDA-MB-231 breast cancer cell line is designated triple negative and characterized as a basal subtype (31, 32). As in the other 3 breast cancer cell lines, LY364947 treatment prior to irradiation strongly radiosensitized MDA-MB-231 cells (Fig. 3A). Prior studies showed that compromised DDR exhibited by *Tgfb1* null mouse epithelial cells or following inhibition of TGFβ signaling in nonmalignant human cells is due to significantly reduced ATM kinase activity (17, 19). ATM directly phosphorylates p53 at serine 15, and indirectly phosphorylates p53 at serine 20 (33). Consistent with increased radiosensitivity, p53 phosphorylation at both serine 15 and 20 was reduced

Table 1. Characteristics of breast cancer cell lines and radiation sensitization

Cell line	Characteristics			TGFβ-mediated growth inhibition at 48 h	Dose enhancement ratio at 10% survival
	Subtype	ER[a]	P53[b]		
MCF10A	Basal B	Neg	WT	35%	1.18
Hs578T	Basal B	Neg	MT	22%	1.04[c]
MDA-MB-231	Basal B	Neg	MT	None	1.26
MCF7	Luminal	Pos	WT	None	1.7
T47D	Luminal	Pos	WT	None	1.26

[a]ER: Estrogen receptor status.
[b]WT: Wild type; MT: Mutant.
[c]DER was estimated based on extrapolation to 10% survival.

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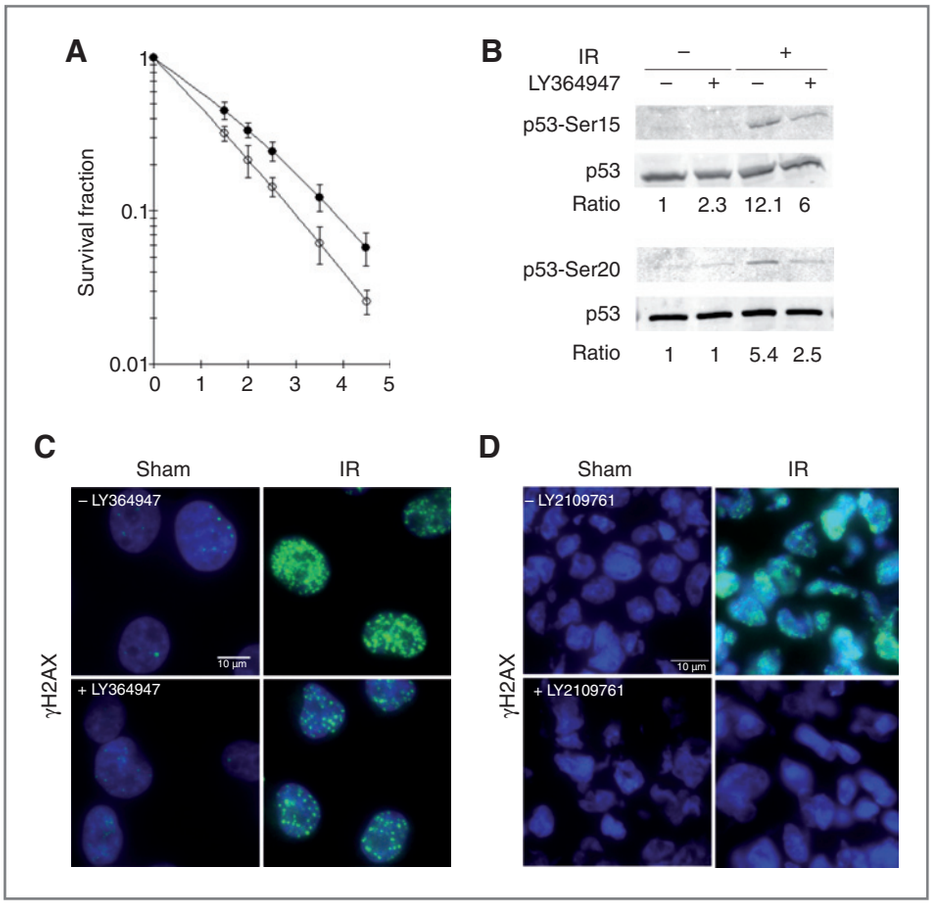

Figure 3. TGFβ inhibition by LY364947 small molecule inhibitor radiosensitizes and attenuates DDR in MDA-MB-231 breast cancer cells *in vitro*. A, MDA-MB-231 cells treated for 48 hours with 400 nmol/L of LY364947 prior to exposure to graded doses of radiation were plated 3-hour postirradiation for clonogenic survival assay. Closed circles (●) represent untreated irradiated controls whereas open circles (○) represent the colony forming efficiency of LY364947 treated, irradiated cells. Mean ± SD values of triplicate determinations are shown for an assay representative of 3 experiments. LY364947 treatment significantly increases radiation sensitivity (ANOVA Tukey test, $P = 0.023$). B, MDA-MB-231 cells treated with LY364947 for 24 hours prior to irradiation with 5 Gy were subjected to immunoblot analysis to examine the effect of inhibiting TGFβ signaling on p53 phosphorylated at serine 15 and at serine 20. TGFβ inhibition prior to irradiation reduces p53 phosphorylation at both sites. The ratio of phosphorylated protein/total protein relative to untreated controls is indicated below each. C, induction of γH2AX foci in cultured MDA-MB-231 cells irradiated with 2 Gy was decreased by pretreatment with LY364947. Cells exhibit nuclear immunostaining of γH2AX foci (green); nuclei are counterstained with DAPI (blue). D, γH2AX foci (green) were reduced in MDA-MB-231 xenograft tumors from mice-treated LY2109761 (bottom) and irradiated with 3 Gy (right). Nuclei are counterstained with DAPI (blue).

in irradiated MDA-MB-231 cells treated with inhibitor (Fig. 3B). ATM also phosphorylates histone H2AX at serine 139, which is called γH2AX, detected as discrete foci at surrounding DNA double-strand breaks caused by ionizing radiation (34). The formation of γH2AX foci is widely used to monitor radiation-induced DNA breaks and to assay DNA rejoining defects (35). Consistent with increased radiosensitivity, p53 phosphorylation at both serine 15 and 20 was reduced in irradiated MDA-MB-231 cells (Fig. 3B). As found in our prior studies, radiation-induced γH2AX foci formation was markedly reduced by pretreatment with LY364947 (Fig. 3C).

To determine whether TGFβ inhibition could similarly affect the DDR *in vivo*, MDA-MB-231 cells were injected in immunocompromised mice to establish xenograft tumors. LY2109761 is a small molecule inhibitor that has similar activity as LY364947 (36), but is more pharmacokinetically stable, is orally available, and is the most potent *in vivo* inhibitor from this family of TGFβ type I receptor inhibitors (37). Mice-bearing MDA-MB-231 tumors were treated with LY2109761 for 2 hours before exposure to 3 Gy. As found *in vitro*, radiation-induced γH2AX foci were decreased in irradiated tumors treated with TGFβ inhibitor, LY2109761 compared with vehicle-treated tumors (Fig. 3D). Smad2 phosphorylation was slightly increased by radiation and both constitutive and radiation-induced Smad2 phosphorylation was decreased by LY2109761 (data not shown).

TGFβ neutralization in combination with radiation increase tumor growth delay

There are 3 pharmacologic routes to blocking TGFβ; neutralizing the ligand, inhibiting expression, or blocking

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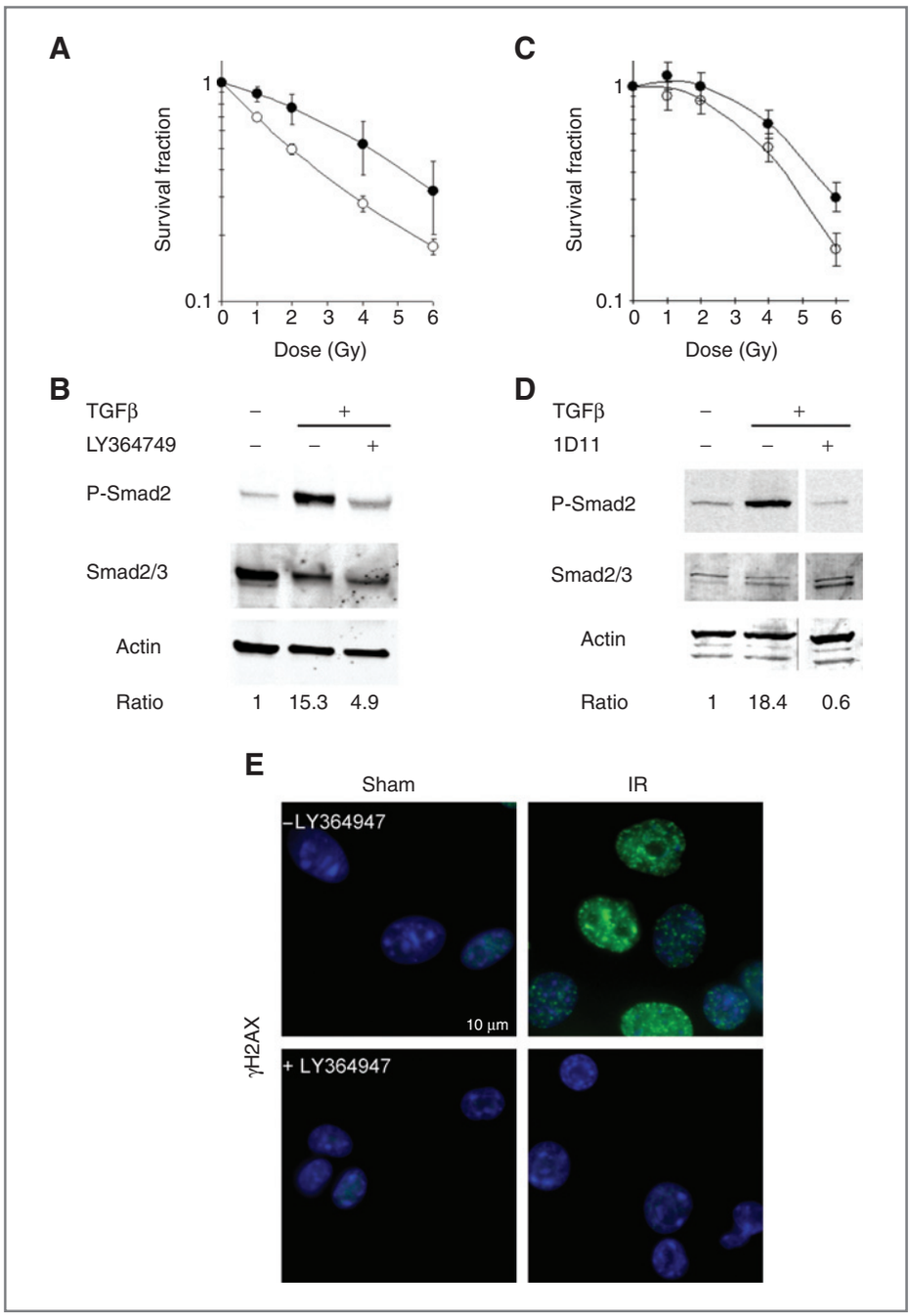

Figure 4. TGFβ neutralizing antibody 1D11 radiosensitized murine mammary cancer cells *in vitro*. A, colony forming efficiency of cells treated for 48 hours with LY364947, prior to irradiation. Closed circles (●) represent control cells whereas open circles (○) represent the colony forming efficiency of LY364947 treated irradiated cells irradiated to the indicated dose. Radiation sensitivity was increased by LY364947 treatment ($P = 0.024$, ANOVA Tukey test). A representative experiment of 3 is shown. B, immunoblots of phospho-Smad2 and total Smad2/3 from 4T1 cells treated with LY364947 for 24 hours followed by TGFβ for 30 minutes or sham treated. The ratio of phosphorylated protein/total protein normalized to control is indicated below each. C, colony forming efficiency of cells treated for 24 hours with 1D11 or 13C4 antibody control, prior to irradiation. Closed circles (●) represent control antibody-treated cells whereas open circles (○) represent the colony forming efficiency of 1D11 TGFβ neutralizing antibody-treated irradiated cells irradiated to the indicated dose. Radiation sensitivity was increased by 1D11 treatment ($P = 0.03$, ANOVA Tukey test). A representative experiment of 3 is shown. D, immunoblots of phospho-Smad2 and total Smad2/3 from 4T1 cells treated with 1D11 for 24 hours followed by TGFβ for 30 minutes or sham treated. The ratio phosphorylated protein/total protein normalized to control is indicated below each. E, 4T1 cells treated with LY364947 for 48 hours prior to irradiation with 2 Gy were immunostained for γH2AX (green) and DAPI (blue). Radiation-induced γH2AX foci were decreased by LY364947TGFβ inhibition.

signaling (36). The pharmacokinetic properties of antibody and small molecule kinase inhibitors result in considerable differences in the duration of TGFβ signal modulation. At this point, the half-life of the TGFβ type I receptor kinase small molecule inhibitors may not be optimal for use in the context of fractionated radiotherapy. Alternatively, several TGFβ neutralizing antibodies are in clinical development that have shown safety and efficacy in fibrotic disorders (36, 38). We compared the efficacy of TGFβ ligand capture using 1D11 pan-TGFβ neutralizing antibodies to LY364947 *in vitro* using murine 4T1 breast tumor cells. Radiosensitization following 1D11 pretreatment (Fig. 4C) was comparable with that following LY364947 pretreatment (Fig. 4A), which indicated that TGFβ ligand sequestration and inhibition of the type I receptor kinase are functionally similar. As expected, both neutralizing antibody and small molecule inhibited Smad 2 phosphorylation activation in response to TGFβ

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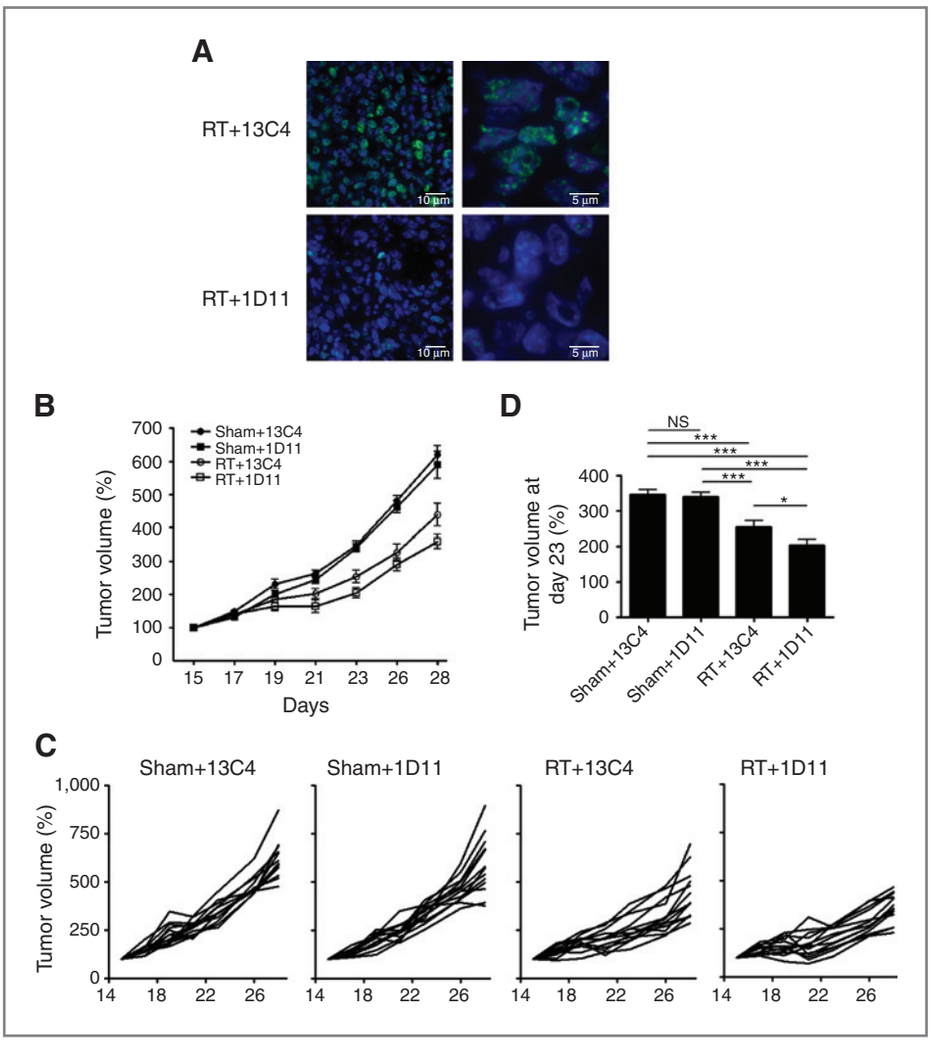

Figure 5. TGFβ neutralizing antibody 1D11 increases radiation-induced tumor delay growth *in vivo*. A, 4T1 tumors treated i.p. with 5 mg/kg 1D11 or 13C4 control antibody and irradiated at 8 Gy and harvested 1 hour later were immunostained for γH2AX (green) and DAPI (blue). γH2AX foci were decreased in tumors from mice treated with 1D11 (bottom) compared with the tumor treated with 13C4 (top). Low (left) and high (right) magnifications are shown. B, 4T1 tumor volumes from mice treated as above on day 13 with 13C4 control antibody (circles) or 1D11 TGFβ neutralizing antibody (squares) with (open symbols) and without (closed symbols) radiation (8 Gy) at day 14. 1D11 treatment increased the response to radiation ($P = 0.01$) but did not itself affect tumor growth rate ($P = 0.77$). C, individual tumor growth curves according to treatment. D, tumor growth delay compared with control was significantly increased by radiation and combination at day 23. $n = 13$ mice/group; *, <0.05; **, <0.01; ***, <0.001; NS, not significant.

(Fig. 4B and D). Moreover, γH2AX foci were significantly reduced in irradiated tumors from mice treated with 1D11 antibodies compared with those treated with 13C4 control antibodies (Fig. 4E).

TGFβ is a key contributor to the immunosuppressive tumor environment (39); radiation-induced TGFβ may hinder the development of anti-tumor immune responses that can be elicited by radiotherapy (40). Thus, to determine the effect of 1D11 antibody in conjunction with radiation treatment, we used 4T1 to establish preclinical breast tumors in immunocompetent mice. Mice-bearing 4T1 subcutaneous tumors were treated with TGFβ neutralizing antibody, 1D11, or a control antibody, 13C4, at 5 mg/kg, i.p. Tumors were sham-irradiated or irradiated with a 8 Gy single dose 24 hours later. Some tumors were randomly selected for harvest 1 hour after irradiation to assess γH2AX foci. As found with MDA-MB-231 cells treated with the small molecule inhibitor LY2109761, γH2AX foci were reduced in irradiated tumor treated by pan-specific antibody against TGFβ *in vivo* (Fig. 5A).

Although 4T1 tumor growth can be inhibited by TGFβ blockade when treated 1 day posttumor inoculation due to an effect on tumor-promoting immune cells (41), the growth rate of established tumors was not affected by TGFβ neutralizing antibody administered at this dose. As expected, irradiated tumors showed a significant tumor growth delay compared with unirradiated tumors (Fig. 5B and C). Tumor growth delay was further increased in mice that were both irradiated and treated with 1D11 neutralizing antibody compared with those that received radiation and 13C4 control antibody (Fig. 5D), with a significantly smaller average tumor volume ($P < 0.05$). No difference was observed with 1D11 treatment alone compared with no treatment controls ($P = 0.77$).

We speculated that increasing TGFβ antibody (50 mg/kg) in the context of multiple radiation fractions (3 × 12 Gy) could possibly provide greater benefit (Fig. 6A). Even at this high dose, a single administration of 1D11 alone in established tumors did not significantly reduce 4T1 tumor growth ($P = 0.16$). Tumor growth rate was profoundly

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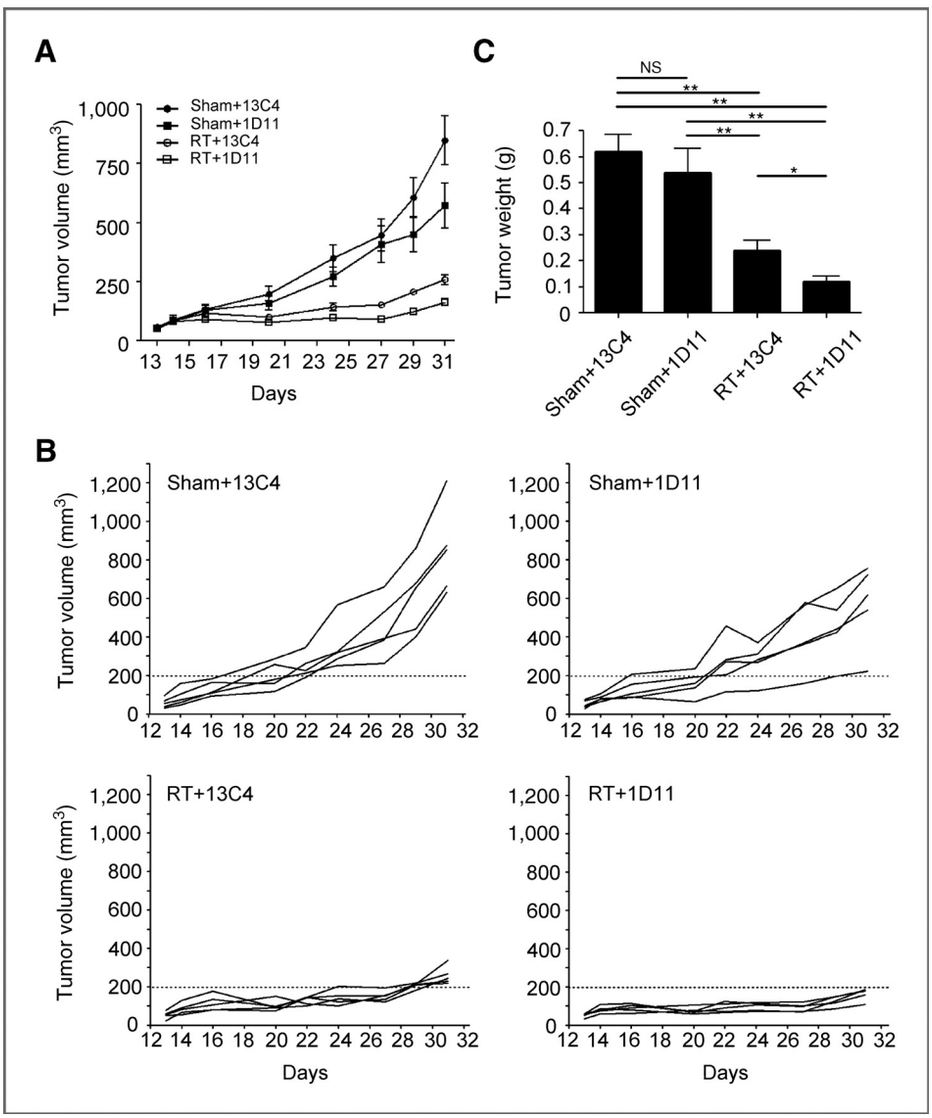

Figure 6. TGFβ neutralizing antibody 1D11 treatment increases the response to multiple fractions of radiation *in vivo*. A, 4T1 tumor volume growth curves from mice treated i.p. on day 13 with 50 mg/kg 13C4 control antibody (circles) or 1D11 TGFβ neutralizing antibody (squares). Tumors were treated with 3 fractions of 12 Gy (open symbols) initiated at day 14 for 3 consecutive days. 1D11 and radiation decreased tumor growth rate compared with radiation and 13C4 ($P < 0.05$). 1D11 treatment alone did not significantly affect tumor growth rate ($P = 0.16$). B, individual tumor growth curves according to treatment. C, tumor weight at experiment termination was significantly decreased by the treatment combination. $n = 5$ mice/group; *, <0.05; **, <0.01; ***, <0.001; NS, not significant.

inhibited by treatment with multiple radiation fractions and further reduced by addition of 1D11 (Fig. 6B) compared with 13C4 ($P < 0.05$). Tumor weight at experiment termination (day 30) was also significantly reduced ($P < 0.05$) by combination treatment (Fig. 6C). No detectable toxicity, as evidenced by mouse husbandry or weight, was noted in mice treated with 1D11 antibodies for either dose (5 or 50 mg/kg) alone, or in combination with localized tumor irradiation (data not shown).

Discussion

We report herein that TGFβ inhibition increased radiation sensitivity measured by clonogenic assay of breast cancer cells *in vitro* and tumor growth delay *in vivo*. As previously shown for chemotherapy (24), inhibiting TGFβ promotes tumor response to radiation therapy. Our prior research using mouse and human epithelial cells showed that TGFβ inhibition compromises radiation-induced ATM kinase activity and downstream effectors of the DDR, resulting in increased cell killing measured by clonogenic assay (19). All 4 human breast cancer cells and a murine tumor cell line were radiosensitized independent of sensitivity to TGFβ-mediated growth inhibition, suggesting that this strategy would be effective across breast cancer subtypes.

Consistent with our earlier studies indicating diminished DDR following radiation exposure, TGFβ inhibition prior to irradiation also resulted in reduced phosphorylation of H2AX and p53 in cultured triple-negative MDA-MB-231 breast cancer cells. Either human MDA-MB-231 xenografts or murine 4T1 tumors in mice treated with TGFβ inhibitors prior to radiation exposure *in vivo* exhibited less γH2AX foci formation, a nuclear marker of the rapid molecular

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radiation response. Moreover, 1D11 neutralizing antibodies enhanced the tumor growth delay after a single radiation exposure, which is consistent with a direct effect on radiosensitvity due to compromised DNA damage recognition. TGFβ inhibition before radiation treatment was most effective in combination with fractionated radiation therapy. Notably, 1D11 antibody did not by itself affect tumor growth rate when administered to established tumors. It is quite conceivable that effects on angiogenesis (37) and immunity (42) could also contribute to greater efficacy of TGFβ inhibition in combination with a fractionated course of radiation.

Teicher and colleagues showed that tumors secreting high levels of TGFβ are more resistant to chemotherapies such as cis-platinum (43, 44). Cis-platinum treatment of MDA-MB-231 breast cancer cells increased both TGFβ mRNA levels and the secretion of active TGFβ, leading to growth arrest and repair of damage: as a result cells became more resistant to cis-platinum killing (43). Anti-TGFβ antibodies enhanced cis-platinum induced DNA fragmentation in MDA-MB-231 cells, restoring cellular sensitivity to cis-platinum (44). Similarly, treatment of animals bearing cis-platinum–resistant tumors with TGFβ neutralizing antibody or with the TGFβ inhibitor, decorin, restores drug sensitivity of the tumor (43, 45, 46). The molecular mechanism(s) underlying this phenomenon remain to be elucidated; in our studies, TGFβ signaling needs to be inhibited before DNA lesions occur to induce radiosensitivity (19). Inhibition of TGFβ type II receptor by halofuginone was shown by Cook and colleagues to enhance the radiosensitivity of diverse tumor cell lines (47).

Exposure to either whole body or localized radiation induces TGFβ production and activation, which in turn modulate late tissue effects (reviewed in ref. 8, 11, 16). Circulating TGFβ was doubled 2 weeks after 4T1 tumor irradiation (Bouquet and Barcellos-Hoff, unpublished data). This persistent elevation could have deleterious consequences. Arteaga and colleagues showed that a radiation-induced systemic surge of TGFβ-promoted metastatic spread (4). In these studies, irradiating nontumor-bearing sites of MMTV/PyVmT transgenic mice increased circulating levels of TGFβ, circulating tumor cells, and lung metastases; these effects were all abrogated by administration of a neutralizing TGFβ antibody. Reiss and colleagues identified antiangiogenic effects as an additional benefit of TGFβ neutralizing antibody mediated by control of VEGF (48). Additional effects of TGFβ neutralization on antitumor immunity (49) and decreased cell cooperation that produces a "protumor environment" (50) could also contribute to benefit in cancer therapy.

Given the established protumorigenic role of TGFβ, its involvement in the response of cancer cells to treatment, and evidence that radiation-induced TGFβ expression and activation lasts a period ranging from weeks to months in irradiated normal tissue (8), the concept of enhanced therapeutic index of radiotherapy from TGFβ inhibition is further supported by our *in vitro* and *in vivo* studies. Of particular interest is that either abrogation of TGFβ signaling by type I receptor kinase small molecule inhibitors or by ligand capture using neutralizing antibodies increased the radiosensitivity of diverse cancer cell lines *in vitro* and impaired DDR *in vivo*, which is the objective in the development of other therapies targeted to the DDR pathway (19, 20). Both MDA-MB-231 and 4T1 are models of triple-negative breast cancer, which has a poor prognosis. Recent studies from Reiss and colleagues show that TGFβ inhibition alone effectively reduce metastasis in a similar setting (37), which lends additional credence to the use of TGFβ inhibition in cancer therapy despite its tumor-suppressor activity. Our demonstration that short-term TGFβ neutralizing antibody increased tumor growth delay after a single radiation exposure and compromised tumor growth rate after fractionated radiation treatment in a syngeneic preclinical model without evidence of associated toxicity provides an additional route to therapeutic benefit. Our data argue for harnessing TGFβ inhibitors at the time of radiotherapy could translate protumor TGFβ biology into clinical benefit.

Disclosure of Potential Conflicts of Interest

M.H. Barcellos-Hoff has an unlicensed patent on the use of TGFβ inhibitors in radiotherapy. The other authors disclosed no potential conflicts of interest.

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Appendix 3:

Formenti, S, Encouse, G, Adams, S, **Pilones, KA**, Ruocco, MG, Dustin, M and Sandra Demaria, Role of T-Lymphocytes for Tumour Response to Radiotherapy *Eur J Cancer*; 47(1); S11.

34 INVITED

Penile Cancer − Chemotherapy

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Penile cancer is a rare disease and accounts for only about 0.5% of all malignancies. Advanced (T3/4) or metastatic disease is even rarer, comprising only 5% of patients in Europe and up to 13% in Brazil.
The role of chemotherapy in the treatment of penile cancer is limited. For patients with fixed or relapsed inguinal nodes upfront combination chemotherapy followed by surgery is recommended. In the adjuvant setting chemotherapy should be considered for patients with pN2/3 disease, although supporting data is scarce. Combination chemotherapy can provide palliation in the case of metastatic disease or relapse.
There are only very small retrospective series and very rare prospetive trials with multiple chemotherapy regimens and partly conflicting results. Since the late 1980-ies the following compounds have been used as single agents, but mostly in combination: methotrexate, bleomycin, cisplatin, 5-fluorouracil (5-FU), vinblastin. More recently the taxanes, irinotecan and ifosfamide have been added to the chemotherapeutic armamentarium. Cisplatin combination chemotherapy is active in penile cancer with response rates of about 20%. The highest response rate of 32% was reported in one of the larger series from the South West Oncology Group with one oft he regimens of the 1990ies (methotrexate, bleomycin, cisplatin). However, toxicity was very high with five treatment related deaths. Cisplatin has become the basis of chemotherapy combinations in more recent series, mostly combined with 5-FU, which is the recommended combination in the European guidelines. The EORTC conducted one of the rare prospective trials and explored the efficacy and safety of cisplatin and irinotecan (Theodore et al, Ann Oncol 2008). The response rate was 31%, including two complete pathologic responses (pCR). Neoadjuvant paclitaxel, ifosfamide, and cisplatin showed an objective response rate of 50%, including three pCR, and acceptable toxicity in a 30 patient prospective trial (Pagliaro et al, J Clin Oncol 2010). The inclusion of the taxanes and contemporary chemotherapy support add to the efficacy of chemotherapy and the reduction of toxicity in the treatment of locally advanced and metastatic penile cancer.

Special Session (Sat, 24 Sep, 14:15−15:15) **Immune System and Tumour Response to Radiotherapy**

35 INVITED

Role of T-Lymphocytes for Tumour Response to Radiotherapy

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Over the past ten years we have developed a clinical translational program based on the rationale of immunizing patients against their own tumour by concomitantly: 1) removing existing “breaks“ in their immune system and 2) harnessing local ionizing radiation (IR) to induce physical and biological perturbations at the irradiated tumour site, to achieve the successful conversion of the original tumour into an immunogenic hub (Formenti, Lancet Oncology 2009). Preclinical investigations have shed some light on the specific role of T cells in these processes. For instance, in the 4T1 syngeneic murine model of metastatic breast cancer targeting regulatory receptors or cells (Treg) by anti-CTLA-4 and anti-CD25 antibodies, respectively, synergized with IR and reduced the number of metastases to the lung (an abscopal effect, defined as a significant growth inhibition of the tumour outside the irradiated field) in a CD8+ T cells dependent way. In the same model IR increased the migration of CD8 CXCR6 activated T cells to tumours. This effect was mediated by IR-enhanced secretion by cancer cells of CXCL16, a chemokine that binds to CXCR6 on Th1 and activated CD8 effector T cells. CXCR6-deficient mice showed reduced infiltration of tumours by activated CD8+ T cells and impaired tumour regression following treatment with local IR + CTLA-4 blockade.
Interestingly, an abscopal effect, occurred only in mice treated with the combination of 9H10 and fractionated radiotherapy, but not when a single dose of 20 Gy was administered ($P < 0.01$), as reflected by the frequency of CD8+ T cells showing tumour-specific IFN-gamma production.
The contribution of invariant natural killer T (iNKT) cells, a subset with unique regulatory functions, in the response to IR and CTLA-4 blockade was also studied. Growth of 4T1 primary tumours and lung metastases was compared in wild type (WT) and iNKT cells-deficient (iNKT−/−) mice. The response to IR+CTLA-4 blockade was markedly enhanced in the absence of iNKT cells: 50% of iNKT−/− compared to none of the WT mice had complete tumour regression, long-term survival, and resistance to a challenge with 4T1 cells.
Finally, intravital microscopy demonstrated that while both IR and CTLA-4 blockade given as monotherapy enhanced the motility of activated CD8 T cells infiltrating 4T1 tumours, IR with anti-CTLA-4 increased the arrest of T cells in contact with tumour cells. The latter required interaction of NKG2D on CD8+ T cells with its ligand retinoic acid early inducible-1 (Rae-1) on the tumour cells, which was up-regulated by IR. Blocking NKG2D-Rae-1 interactions increased markedly the motility of anti-CTLA-4 treated T cells within irradiated tumours inhibiting their contact with tumour cells, and abrogated immune-mediated tumour rejection, suggesting a critical role of radiation-induced NKG2D ligands for the antitumour effects of anti-CTLA-4 in the setting of a poorly immunogenic tumour.

36 INVITED

CD11b Cells Provide Resistance to Radiotherapy

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We are testing a new therapeutic paradigm based on the dual origin of tumour blood vessels: Angiogenesis, the sprouting of endothelial cells from nearby blood vessels, and vasculogenesis, the formation of blood vessels by circulating cells, primarily of bone marrow origin. We have shown that by killing the endothelial cells in and surrounding the tumour, local tumour irradiation abrogates local angiogenesis suggesting that the tumour has to rely on the vasculogenesis pathway for regrowth after irradiation. We have shown that local irradiation of human tumour xenografts in nude mice produces a large influx of bone marrow derived CD11b+ myelomonocytes into the tumours as they begin to regrow following irradiation. We demonostrate that inhibition of this influx using neutralizing antibodies against CD11b inhibits tumour recurrence. Thus the influx of CD11b+ monocytes promotes tumour recurrence after irradiation. The mechanism for this effect could be by their proangiogenic nature or they could be suppressing T-cell immunity by their nature as myeloid-derived suppressor cells (MDSC). The fact that these experiments were performed in T-cell deficient mice does not rule out the MDSC mechanism as we and others have demonstrated that there is residual anti-tumour immunity in nude mice. To distinguish the two mechanisms we also tested anti-Gr1 antibodies and showed no effect on tumour response to irradiation. As MDSC are Gr1+CD11b+ monocytes these data argue for the importance of the proangiogenic properties of Gr1− CD11b+ cells. We are testing other models including immunodeficient SCID mice to further interrogate the mechanism by which CD11b+ myelomonocytes promote tumour recurrence after irradiation.

Special Session (Sat, 24 Sep, 14:15−15:15) **Developments in Surgical Oncology**

37 INVITED

Improving the Diagnostic Pathway for Men With Prostate Cancer

Abstract not received

38 INVITED

Robotic Surgery − Opportunities and Issues for Nursing

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The operating theatre of the 21st century has become a hi-tech environment. Since the early days of laparoscopic surgery, there has been a continuous increase in the number of devices for surgical use thus, crowding of the operating theatre.
Robotic surgery is quickly replacing conventional surgery in several surgical specialties and is not only heralded as the new revolution, but is one of the most talked about subjects in surgery today. Such advances have facilitated significant improvements in the management of the surgical patient effectively cancer patients, minimising open surgical resections.
Results have shown that robotic procedures reduce recovery times in addition to a shorter hospital stay, reduced pain, reduced tissue damage, and scarring. This change bears a significant impact on the clinical practice of surgeons, surgical trainees and operating theatre practitioners.
In September 2000 the da Vinci Robotic System, the first of its kind to be installed in the UK, was introduced to Imperial College St Mary’s Hospital London. The role of the robotics nurse specialist was developed to create